POTENTIAL APPLICATIONS OF BIOSURFACTANT FROM MARINE BACTERIA IN BIOREMEDIATION

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT
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MASTER OF SCIENCE IN LIFE SCIENCE

BY

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CERTIFICATE

This is to certify that the project report titled **"Potential applications of biosurfactant from marine bacteria in bioremediation"** submitted by **Ms. Manorama Mohanty** to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in LIFE SCIENCE is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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Dedicated to my beloved Parents and

my Sisters

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Date: 10.05.2013 Manorama Mohanty

Place: Rourkela Roll no. 411LS2054

DECLARATION

I hereby declare that the thesis entitled **"Potential applications of biosurfactant from marine bacteria in bioremediation"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Surajit Das, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge no part of this thesis has been submitted for any other degree or publication in any form.

Date: Manorama Mohanty

Place: Rourkela Roll no. 411LS2054

LIST OF SYMBOLS AND ABBREVIATIONS USED

g	Gram
h	Hour
l	Litre
µl	Micro litre
º	Degree
ml	Millilitre
C	Centigrade
min	Minute
LB	Luria Bertani
BHB	Bushnell Haas Broth
%	Percentage
+ve	Positive
-ve	Negative
PAHs	Polycyclic Aromatic Hydrocarbons
no.	Number
v	Volume
w	Weight
CMC	Critical micelle concentration
MEOR	Microbial enhanced oil recovery
HPLC	High performance liquid chromatography

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ABSTRACT

Marine bacteria were screened for their ability to synthesize biosurfactants which can effectively degrade polycyclic aromatic hydrocarbons (PAHs) as the sole carbon and energy source. PAHs are very harmful to flora and fauna having many toxic effects and also affecting mankind adversely. The main objective of this work was to study the potential applications of biosurfactant in aerobic degradation of PAHs in stress conditions. The antimicrobial and anti-adhesive studies of the biosurfactant were also tested against different pathogenic strains. Marine bacteria were isolated from sediment samples of Paradip Port, Visakhapatnam Port, Rishikulya, Bhitarakanika and screened for their biosurfactant production and also growth was optimised in carbon and nitrogen sources for the best biosurfactant production. Naphthalene and Phenanthrene (PAHs) degrading isolates were evaluated for their biodegradative potential under laboratory scale through UV-Vis spectroscopy, phenotypical characterization by SEM studies. Five potent isolates JV201, JV501, JV502, JP011, and JP022 were identified to be *Ochrobactrum, Streptococcus, Pseudomonas sp*., *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* having 99.9%, 99.6%, 99%, 99.3%, 98.6% of degradation of Phenanthrene (100mg/l) and 99%, 99.1%, 89.75%, 94.01%, 97.02% of degradation of Naphthalene (100 mg/l) respectively having good antimicrobial and anti-adhesive properties.

Keywords: Biosurfactants, PAHs (polycyclic aromatic hydrocarbons), Bioremediation

1. INTRODUCTION

Marine environment is the largest habitat as compared to other habitat in the biosphere. About 70% of the earth surface is covered by salt water. It is believed that the life is originated first from ocean. Industrialization and extraction of natural resources have resulted in large scale environmental contamination and pollution. Large amounts of toxic wastes have been dispersed in thousands of contaminated sites spread across our nation whose common sink are the coastal marine regions. Thus every one of us is being exposed to contamination from past and present industrial practices, emissions in natural resources (air, water and soil) even in the most remote regions. The risk to human and environmental health is rising and there is evidence that this cocktail of pollutants is a contributor to the global epidemic of cancer, and other degenerative diseases. These pollutants belong to two main classes: inorganic and organic. The challenge is to develop innovative and cost-effective solutions to decontaminate polluted environments, to make them safe for human habitation and consumption, and to protect the functioning of the ecosystems which support life. The pollution is mainly by various anthropogenic sources like oil spill, pesticides, plastic debris, fertilizers, chemicals, heavy metals, radioactive substances, biological, solid wastes. Nutrient pollutions-eutrophication, algal bloom (*brevitoxin*, *ciguatera toxin*, *cyanobacteria*), is due to the use of land as agricultural and industrial development. Sound pollution in ocean is due to shipping, sonar system, seismic exploration, chemical and nuclear explosions, bring threats to marine lives. Heavy metals like mercury, lead, cadmium, and zinc all are very toxic and they are taken by the organisms leading to biomagnifications increasing their toxicity to the subsequent tropic levels. For example, the methylated mercury present in the sea organisms enter in to the human body by food (fish, shellfish etc.) which adversely affects mankind. Oil from the airplanes, individuals vehicles like car, jet skis, mowers contribute to the oil pollution in the ocean by land runoff. Oil spill is a major pollution in marine environment. About 38 super tankers have taken place in the last five decades. PAHs (polycyclic aromatic hydrocarbons) are the major constituent of oil. The marine organism- fish, oysters, crab, and shrimp, they can't avoid the oil as they are living in the water. The seabirds unable to catch fish as their feathers are coated by the oil. The oil spill also creates the problem to control the body temperature in the marine animals and cause pneumonia. Some are dying due to the shortage of dissolved oxygen. Both coral reefs and mangroves contribute a lot to the marine ecosystem, but due to these changes in original elemental concentrations, they fail to cope with the polluted environment. The oil spill has the physical, psychological, genotoxic and

endocrine effects in the human body. Many fishermen and harvesters also are adversely affected leading to various health problems.

PAHs are the large group of organic compounds, formed by the hydrogen and carbon atoms with two or more fused benzene rings. These are also called polycyclic aromatic hydrocarbons or poly nuclear aromatic hydrocarbons. These are the hydrophobic compounds and insoluble in water. More than 1,000 types of PAHs compounds are present in the environment with differing in the number and position of aromatic rings. Most of them are carcinogenic, mutagenic and teratogenic to many organisms including mammals. PAHs are found everywhere in the nature for example soil, air, water, flora and fauna.

Physical and chemical characteristics of PAHs generally vary with molecular weight. With increasing molecular weight, aqueous solubility decreases and melting point, boiling point, and the log K_{ow} (octanol/water partition coefficient) increases (Table 1) suggesting increased solubility in fats, a decrease in resistance to oxidation and reduction, and a decrease in vapour pressure. Accordingly, PAHs of different molecular weight vary substantially in their behaviour and distribution in the environment and in their biological effects.

Table 1: Some selected PAHs with their physical and chemical properties

Compound	**No of rings**	**Approximate molecular weight**	**Melting point (˚C)**	**Water solubility (mg/l)**	**Log K_{ow}**
Naphthalene	2	128	80	30.0	3.37
Phenanthrene	3	178.23	99	insoluble	4.57
Biphenyl	2	154.20	69-71	insoluble	4.09
Anthracene	3	178	216	0.07	4.45
Benz(a)Anthracene	4	228	158	0.014	5.61
Benzo(a)Pyrene	5	252	179	0.0038	6.04
Benzo(g, h, i) pyrene	6	276	222	0.00026	7.23

1.1. Sources of PAHs pollution in the environment

PAHs are produced due to the incomplete combustion of organic substances (fossil fuels). An oxygen deficient flame and temperature in between 650˚C-900˚C are the suitable conditions for PAHs production. The sources of production of PAHs are mainly divided in to two types. These are natural sources and anthropogenic sources. Somehow the natural sources like forest fires and volcanoes are also contribute to the total PAHs production in the environment. Anthropogenic sources produce more amounts of PAHs compounds than natural sources. Anthropogenic sources can be categorized in to two types. One is combustion of materials for energy supply like coal, oil, gas, wood etc and another one is combustion for waste mineralization or waste incineration. The first one includes mainly coke and carbon production, crude oil, petroleum product (such as diesel fuel, kerosene, lubricating oil and asphalt), wood, coal, and mobile sources like cars, trains, airplanes and sea traffic. Incineration of industrial and municipal wastes is included in the second one (Maliszewska-Kordybach, 1999). Other sources are like agricultural burning, cigarette smoking, volatilations from vegetation and soil, chlorophyllous and non chorophyllous (bacteria and fungi). Traffic pollution is also a major source of PAHs present in the atmosphere. As these are the hydrophobic compounds they combine with the organic and inorganic particles rapidly. Then these are washed from the roads during rainfall and enter in to the natural environment (Ball et al., 1991).

1.2. Source of PAHs pollution in Marine environment

Every year, about 43,000 metric tons of PAHs are entering in to the atmosphere and 23,000 metric tons are released to the marine environment by anthropogenic sources (Eisler, 1987). PAHs may enter in to the marine environment by spillage of petroleum and petroleum products, atmospheric deposition of PAHs, domestic and industrial sewages, surface land runoff, biosynthesis (Fig. 1 and Table 2). PAHs associated with some airborne particles settle down in bottom of the sea. The petroleum and petroleum products undergo dispersion, evaporation, some chemical changes, sunlight effect (photo-oxidation) (www.intechopen.com/download/pdf/29372).

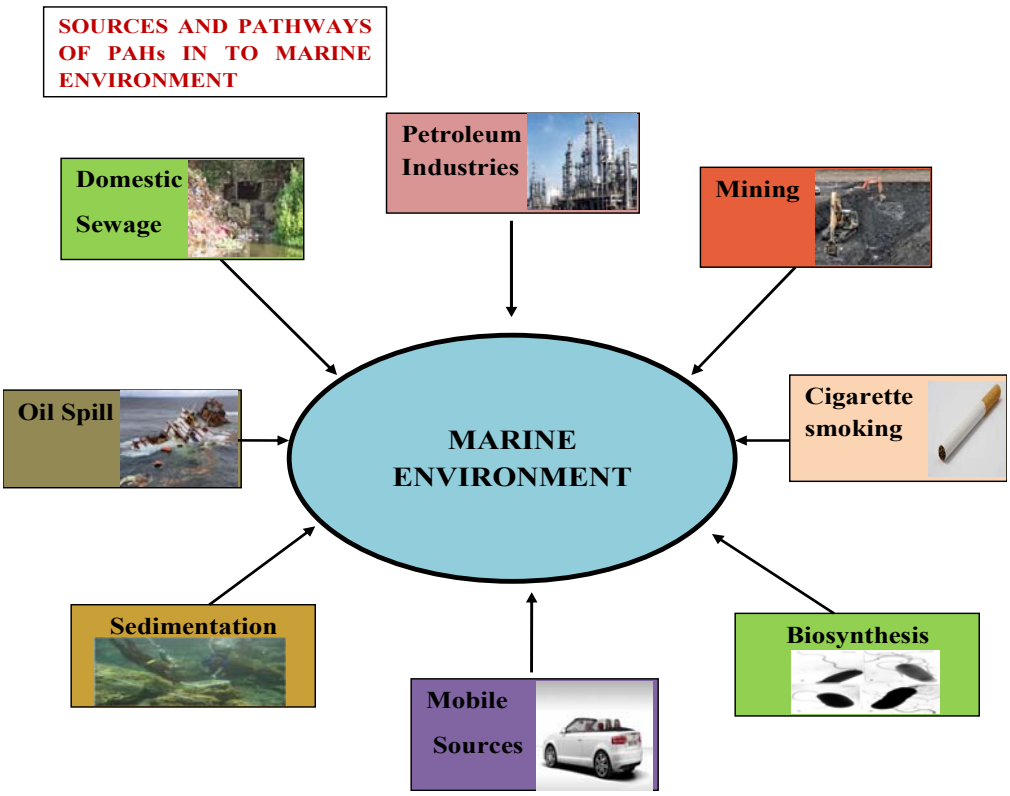


Fig. 1: Sources and Pathways of PAHs into marine environment.

Table 2: Main sources of PAHs in Aquatic environment (Eisler, 1987)

Sources	Annual inputs of total PAHs
Petroleum spillage	170,000
Atmospheric deposition	50,000
Waste water	4,400
Surface land runoff	2,940
Biosynthesis	2,700
Total	230,040

1.3. Fate of PAHs in environment

PAHs are well known for their existence in the environment and carcinogenicity property. In the past, there is a balance in PAHs production and degradation in nature due to microbial transformation and photo oxidation. But now it is disturbed due to the increase in the development of the industries and use of fossil fuels as energy source. PAHs associate with various particles in the atmosphere. They are transported to various regions and also they are persisted in the environment by depending upon their size, meteorological conditions and

atmospheric physics. Some days or some weeks are required to degrade the PAHs; those are highly reactive with the ozone layer (photo-decomposition) and various oxidants in the atmosphere. PAHs associate with smaller particles are easily inhaled (Lee and Grant, 1981) and they also cause various problems in birds, insects and bats living in the air. PAHs can form different mutagenic and carcinogenic products by the process photo oxidation (Fig. 2) and it is also a bioremediation process of removal of PAHs in the atmosphere (Edwards, 1983). Chemical and photochemical modifications of PAHs in the atmosphere can occurred due to light intensity, concentration of gaseous pollutants (O_3, NO_x, SO_x). The half life of benzo(a)pyrene in the atmosphere is 10 min to 72 days (Valerio et al., 1984). PAHs deposited in the soil by directly or indirectly by vegetation. Some plants adsorb the PAHs in their leaves, by which they enter in to the food chains. Plants may photo degrade, metabolize or translocate and accumulate these PAHs in their tissues. PAHs are metabolized by the microbes and animals for energy and carbon source.

ENVIRONMENTAL FATE OF PAHs:

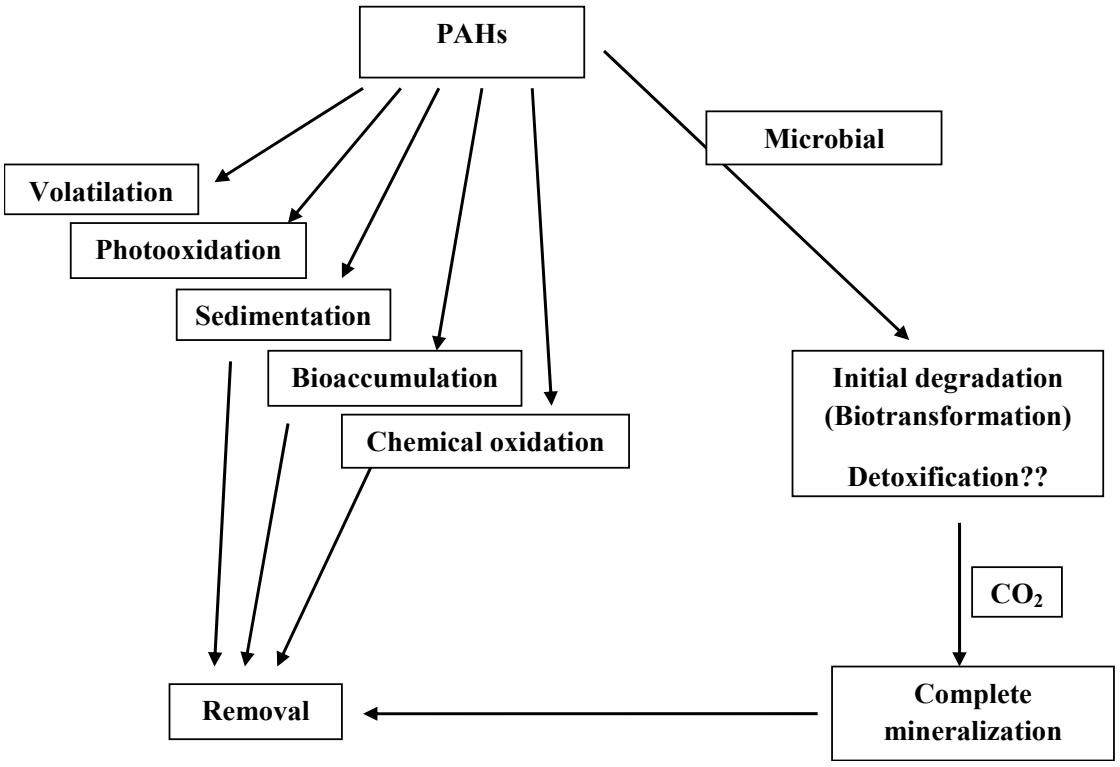


Fig. 2: Schematic diagram showing Environmental fate of PAHs

1.4. Effect of PAHs on Plants, Human and Marine organisms

1.4.1. On Plants

Terrestrial vegetations are also affected by the PAHs. Some PAHs are absorbed by the roots and foliages of some plants. The factors like concentration of PAHs, soil type, solubility in water and the plant species determine the rate of PAHs absorption. Biomagnifications is very less in terrestrial plant. PAHs are accumulated more in plant body those are exposed to atmosphere and very less in underground parts of the plants due to atmospheric deposition. Phytotoxic effects are rare. In the soil PAHs are existed for a long period and transferred to human food chain from soil.

1.4.2. On Human

PAHs are available to human beings in occupational and general environment. There are three ways by which human exposed to PAHs. These are respiratory tract, gastrointestinal tract and skin contact. Non-smoking persons are also exposed to the PAHs due to the diet they are taking like cereals, oils and vegetables. Basically cooked food is the contributor of PAHs in the human body. Also in ground water many PAHs are found: anthracene, pyrene, fluoranthene, anthracene. 1 % of the total acceptable level of PAHs comes from drinking water. About 20-40 mg of benz(a)pyrene is entering in to the body by smoking (Skupinska et al., 2004). Before entering in to the host body PAHs are inactive and can't cause carcinogenesis effect. Tumours can occur in stomach, liver, lungs by BaP and other PAHs. Mastrangelo and co-workers (1996) have given the evidences that lung cancer and bladder cancer are dose dependent and they have done a comparative study between occupationally exposed subjects and truly non-exposed groups. They have given the data that the threshold value 0.2mg/m^3 of benzene soluble matter is not acceptable because it causes relative risk 1.2-1.4 for lung cancer and 2.2 for bladder cancer.

1.4.3. On Marine Organisms

In the hydrocarbon family, PAHs are the most toxic pollutants. In marine environment the toxicity of PAHs varies substantially. In crustaceans the level of toxicity is more and in teleosts it is very low. Some marine plants and animals' uptake the PAHs, then accumulate it. Uptake of the PAHs is highly species specific. PAHs are readily absorbed by the fish and crustaceans whereas some algae and molluscs are unable to metabolize these PAHs. Many marine organisms have the ability to eliminate these PAHs, so biomagnification is not

observed in the food chain (Eisler, 1987). For the biotransformation activity of some organisms, the PAHs concentration decreases with increase of tropic level.

Some PAHs are accumulated in the cell membrane of the microbes due to their lipophilic nature. Due to the exposure to PAHs, the cell damage, carcinogenesis, teratogenesis, and mutagenesis are caused to varieties of organisms as PAHs binds covalently with the macromolecules like DNA, RNA, and protein.

1.5. Microbial Degradation of PAHs

To remove these contaminations from environment, various physico-chemical methods are introduced. But these are too expensive, non specific and also they introduce the secondary contaminants to the environment. So an ecofriendly, cost-effective and bio based method is adapted to treat these contaminations, called bioremediation. Bioremediation is a method of detoxification and degradation of toxic pollutants either through intracellular accumulation or by enzymatic transformation to less toxic or non toxic compounds (Singh et al., 2008). Microorganisms have the potential to degrade, transform or chelate the toxic chemicals, but this transformation process is very slow. The main components of this bioremediation are the microbes and their products. Both the natural and recombinant microbes are used for bioremediation. The important factors of bioremediation are bioavailability of contaminants, availability of microbes and a suitable environment. Also the nutrients, oxygen, pH value, concentration and bioavailability of the contaminants influence the bioremediation of PAHs (Singh Cameotra and Makkar, 2010). Bio-augmentation and bio-stimulation are two processes that enhance the rate of biodegradation by increasing the bioavailability of the pollutant. Bio-stimulation refers to the addition of specific nutrients to a contaminated site with emphasis on the naturally indigenous microbes presumably present in sufficient numbers and types to break down the waste effectively (Scow and Hicks, 2005). Bio-augmentation, an alternative method to bio-stimulation which is the scientific approach to achieve controlled, predictable, and programmed biodegradation. Bio augmentation involves the addition of specifically formulated microorganisms to a contaminated site (Gentry et al., 2004).

Bioremediation of organic compounds is adapted due to their ubiquitous distribution, low bioavailability and high persistence in soil, and their hazardous effect on living creatures. Due to the low water solubility and hydrophobic nature of PAHs, many microorganisms have developed several mechanisms to increase the bioavailability of such compounds in order to utilise them as carbon and energy source. Microbes implement some long term mechanisms

to enhance the bioavailability of such hydrophobic contaminants. These are biofilm formation and biosurfactant production. The method of removing the hydrocarbon contaminants from the environment by using biosurfactant is a viable method.

Biosurfactants are the secondary metabolites produced on living surfaces, mostly on microbial cell surfaces, or excreted extracellularly containing hydrophobic and hydrophilic domains that reduce surface tension (ST) and interfacial tensions between individual molecules at the surface and interface respectively. Microorganisms produce two main types of surface active compounds: biosurfactants and bioemulsifiers. Biosurfactants significantly reduce the air-water surface tension while bioemulsifiers do not reduce as much the surface tension but stabilize oil-in-water emulsions.

Example of some biosurfactants: Rhamnolipid, Sophorolipid, Surfactants, Lipopeptide, Trehalose tetra ester. Biosurfactants in the degradation of heavy metals, polycyclic aromatic hydrocarbons (PAHs), pesticides in soil and water environment, have a great significance. Organic compounds are generally used by the microbes as the source of carbon and energy for their growth. When the carbon source is a hydrocarbon (C_xH_y) or any insoluble substrate, microorganisms facilitate their diffusion into the cell by producing a variety of substances, the biosurfactants. Some bacteria and yeasts excrete ionic surfactants which emulsify the C_xH_y substrate in the growth medium (Karanth et al., 1999). Ex: Rhamnolipids which are produced by different *Pseudomonas* spp. (Guerra et al., 1984), non-ionic trehalose corynomycolates are produced by many *Mycobacterium* spp. and *Arthrobacter* spp.

1.6. Properties of Biosurfactant

There are many advantages of biosurfacants if we compare it with chemically synthesized surfactants. They are biodegradable, generally low toxic, biocompatible, digestible for which they are applied in cosmetics, pharmaceuticals and as functional food additives. They can be produced from cheap raw materials which are available in large quantities. Biosurfactants can also be produced from industrial wastes and by-products and this is of particular interest for bulk production (e.g. for use in petroleum-related technologies). Biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil. They are more effective at extreme temperatures, pH and salinity.

1.7. Biosurfactant Structure, Classification and Characteristics

During the last decades, there has been a growing interest in isolating microorganisms that produce surface active molecules with good surfactant characteristics such as low critical micelle concentration (CMC) and high emulsification activity, simultaneously presenting low

toxicity and good biodegradability. Generally the type and amount of the biosurfactants, produced by microbes depends on the producer organism, factors like carbon and nitrogen, trace elements, temperature. They are also classified into two different categories on the basis of their molecular weight: Low molecular weight biosurfactants and high molecular weight biosurfactants. Examples of low molecular weight biosurfactants are lipopeptides, glycolipids and phospholipids (having lower surface and interfacial tension) and high molecular weight biosurfactants are polymeric and particulate surfactants (more efficient as emulsion-stabilizing agents).

Biosurfactants are categorized mainly by their microbial origin and chemical composition (type of polar group present). According to the studies of Desai and Banat (1997) and Gautam and Tiagi (2005), biosurfactants, based on the structure of their hydrophilic part, are mainly classified in to five categories:

- Glycolipids
- Lipopeptides
- Fatty acids
- Polymer type
- Particulate biosurfactants

Glycolipids

Glycolipids are the most common types of biosurfactants. They are the carbohydrates in grouping with long-chain aliphatic acids or hydroxyaliphatic acids. The constituent mono, di, tri and tetrasaccharides include glucose, mannose, galactose, glucuronic acid, rhamnose and galactose sulphate. The connection is by means of either an ether or ester group. Rhamnolipids, sophorolipids and trehalolipids are the best known glycolipids. The glycolipids can be categorized as:

***Trehalose lipids*:** Microbial trehalose lipid biosurfactants are reported having various structural types. Disaccharide trehalose linked at C-6 and C-6 to mycolic acid. Mycolic acids are the long chain, α-branched and β-hydroxy fatty acids. Cord factors from different species of *Mycobacteria*. *Corynebacteria*, *Nocardia* and *Brevibacteria* differ in size and structure of the mycolic acid esters. Trehalolipids from diverse organisms vary in the size and structure of mycolic acid, the number of carbon atoms present and the extent of unsaturation.

***Sophorolipids*:** Generally various strains of the yeast, *Torulopsis* produce these types of biosurfactants. The sugar unit is the disaccharide sophorose, consists of two β-1, 2-linked glucose units. The sophorolipids reduce surface tensions between individual molecules at the surface, although they are effective emulsifying agents.

***Rhamnolipids*:** This glycolipid (Fig. 3) consists of two molecules of rhamnose and two molecules of β–hydroxydecanoic acid, mainly produced by some *Pseudomonas* spp. While the OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, the OH group of the second acids is involved in ester formation. The production of rhamnose which contains glycolipid was first studied in *Pseudomonas aeruginosa* (Jarvis and Johnson, 1949).

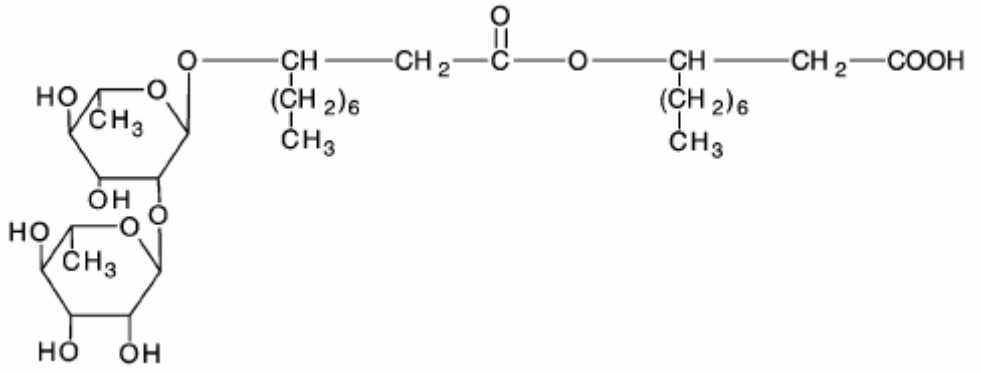


Fig. 3: Structure of Rhamnolipid

Lipopeptides and Lipoproteins

A large number of cyclic lipopeptides including decapeptides antibiotics (gramicidin) and lipopeptides antibiotics (polymyxins) by *Bacillus brevis* and *Bacillus polymyxa* possesses remarkable surface active properties. The cyclic lipopeptide surfactin (Fig. 4) produced by *B. subtilis* ATCC 21332 is one of the most powerful biosurfactants (Arima et al., 1968; Desai and Banat, 1997).

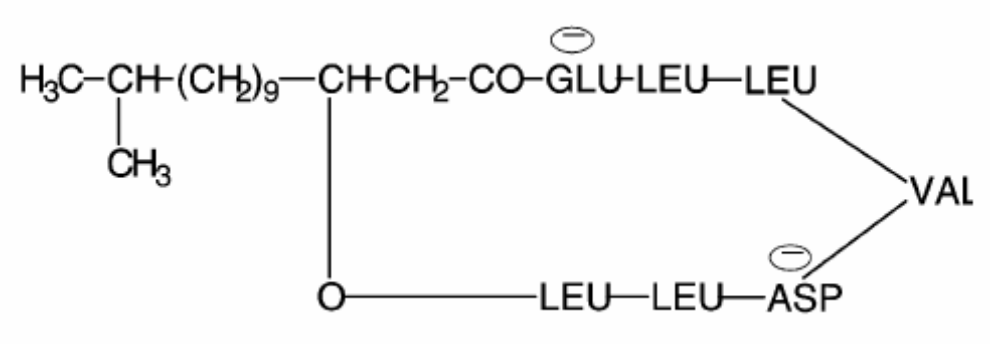


Fig. 4: Structure of Surfactin

Fatty acids, phospholipids and neutral lipids

Large quantities of fatty acids and phospholipids are produced by some bacteria and yeasts during growth on n-alkane (Cirigliano and Carman, 1985). The hydrophilic and lipophilic balance (HLB) is directly proportional to the length of the hydrocarbon chain in their structures (Kretschmer et al., 1982). Phospholipids are the main component of microbial membrane.

Polymeric Biosurfactants

Emulsan, liposan, mannoprotein and other polysaccharide protein complexes are the best studied polymeric biosurfactants. *Candida lipolytica* produce an extracellular water-soluble emulsifier, i.e. Liposan and is composed of 83% carbohydrate and 17% protein (Cirigliano and Carman, 1985).

Particulate Biosurfactants

Extracellular membrane vesicles partitioned hydrocarbons to form a micro emulsion, which plays an important role in alkane uptake by microbial cells. Vesicles of *Acinetobacter* sp. having a diameter of 20–50 nm and a buoyant density of 1.158 cubic g /cm, consists of protein, phospholipids and lipopolysaccharide. The cellular lipid content of *Pseudomonas nautica* increased in eicosanoid-grown cells up to 3.2 fold, compared with acetate-grown cells. Phospholipids, mainly phosphatidyl ethanolamines and phosphatidyl glycerides, were accumulated in eicosanoid-grown cells (Husain et al., 1997).

2. REVIEW OF LITERATURE

Xenobiotics are pollutants that are introduced in to the environment by the direct and indirect action of man. These pollutants include PAHs, oil derivatives, persistent organic pollutants, heavy metals and many others. Among them PAHs are the main and common toxic pollutants. They have harmful manifestations like mutagenic, carcinogenic and genotoxic effects. In contaminated environments, the natural degradation of PAHs can't occur effectively due to their hydrophobicity nature. So they are poorly available to the microorganisms. Biosurfactants make them available to the microorganisms by solubilisation or by emulsification.

2.1. Microorganisms producing different types of biosurfactants

Biosurfactants are the secondary metabolites microbes produced on the cell surfaces (Table 3). The isolation and identification of biosurfactant producers is now increasing day by day (Luna et al., 2013). Some biosurfactants and their respective producers are given in Table 3. There are different types of biosurfactants that are produced from different microbes and they possess different chemical structure. Glycolipids are the most common type of biosurfactants. Some well known glycolipids are rhamnolipid, sophorolipids and trehalolipids (Desai and Banat, 1997; Karanth et al., 1999).

Researchers have mentioned that for the first time *P. chlororaphis* strain produce rhamnolipid by utilizing glucose as the carbon source. The highest level of rhamnolipid produced here is 1g/l (approximately). Also *P. aeroginosa* produces 1.0 to 1.6 g/l rhamnolipid by using glucose (Sim et al., 1997). They used the minimal media for the rhamnolipid production and room temperature as optimum temperature which was the best condition as the strain produce highest rhamnolipid in this condition. But this condition was varied for *P. aeruginosa* for production of rhamnolipid. So a non-pathogenic strain like *P. chlororaphis* can be used commercially for the production of rhamnolipid (Gunther IV et al., 2005).

Rhamnolipids are produced by the genus *Pseudomonas*. They generally contain rhamnose and 3-hydroxy fatty acid (Lang and Wullbrandt, 1999; Rahmaan et al., 2002b). According to Haussler et al. (1998) *Burkholderia pseudomallei* produced a rhamnolipid

which have the haemolytic and cytotoxic properties. *Pseudomonas chlororaphis*, a non-pathogenic strain produce rhamnolipid (Nereus, 2005).

The paper by Vasta et al., (2010) provides an overview of the effect of rhamnolipids in animal and plant defence responses. The current knowledge on the stimulation of plant and animal immunity by these molecules, as well as on their direct antimicrobial properties is also reported. Given their ecological acceptance owing to their low toxicity and biodegradability, rhamnolipids have the potential to be useful molecules in medicine and to be part of alternative strategies in order to reduce or replace pesticides in agriculture.

Researchers have isolated biosurfactant from a marine *Streptomyces* species B3 and studied their properties (Khopde et al., 2012). It is reported here that the biosurfactant production is high in sucrose containing medium and less in glycerol containing medium and the best nitrogen source is yeast extract. At different pH, salinities and temperature, they have studied the emulsification and stabilization of the biosurfactant. The CMC is 110 mg/l and it reduce the surface tension of water is 29mN/m. It also shows a very strong antimicrobial property. For microbially enhanced oil recovery, *Streptomyces* sp. B3 can be used.

Table 3: Microorganisms producing biosurfactant (Kosaric, 2001)

Microorganisms	**Types of Biosurfactants**
Torulopsis bombicola	Glycolipid (sophorose lipid)
Pseudomonas aeruginosa	Glycolipid (rhamnose lipid)
Bacillus licheniformis	Lipoprotein (?)
Bacillus subtilis	Lipoprotein (surfactin)
Pseudomonas sp. DMS 2847	Glycolipid (rhamnose lipid)
Arthrobacter paraffineus	Sucrose and fructose glycolipids
Arthrobacter	Glycolipid
Pseudomonas flurescens	Rhamnose lipid
Pseudmonas sp. MUB	Rhamnose lipid
Torulopsis petrophilum	Glycolipid and/or protein
Candida tropicalis	Polysaccharide-fatty acid complex
Corynebacterium lepus	Corynomycolic acids
Acinetobacter sp. HO1-N	Fatty acids, mono-and diglycerides
Acinetobacter calcoaceticus	Lipoheteropolysaccharide

Rag-1	(Emulsan)
Acinetobacter calcoaceticus 2CAC	Whole cells (lipopeptide)
Candida lipolytica	Liposan (mostly carbohydrate)
Candida petrophilum	Peptidolipid
Nocardia erythropolis	Neutral lipids
Rhodococcus eryithropolis	Trehalose dimycolates
Corynebacterium salvonicum SFC	Neutral lipid
Corynebacterium Hydrocarboclastus	Polysaccharide-protein complex

2.2. Application of biosurfactants in various fields

2.2.1. In pharmaceutics

i) Gene delivery

It is an efficient and a safe method for introducing exogeneous nucleotides in to mammalian cells. In comparision with commercially available cationic liposomes, biosurfactant based liposomes show increasing efficiency of gene transfection.

ii) Anti-cancer activity

In the human promyelocytic leukemia cell line, some microbial extracellular glycolipids induce cell differentiation instead of cell proliferation and exposure of PC 12 cells to MEL enhanced the activity of acetylcholine esterase and interrupted the cell cycle at the G1 phase with resulting overgrowth of neuritis and partial cellular differentiation. This suggests that microbial extracellular glycolipids act as novel reagents for the treatment of cancer cells (Okoliegbe and Agarry, 2012).

iii) Antimicrobial activity

Many biosurfactants have strong antiviral, antibacterial and antifungal activity. Das and co-workers (2009) have reported a biosurfactant produced by marine *B. circulans* that has a potent antimicrobial strains including MDR strains.

iv) In cosmetics

Biosurfactants could be used as antacids, bath products, acne pads, antidandruff products, contact lens, hair colours and care products, deodorants, nail care, lipsticks, lip makers, soap, tooth pastes, baby products, foot care, conditioners, shampoos health and beauty products.

Others are: inhibition the adhesion of pathogenic organisms to solid surfaces, recovery of intracellular products, immunological adjuvant.

2.2.2. In agricultural use

Surface-active agents are needed for the hydrophilization of heavy soils to obtain good wettability and also to achieve equal distribution of fertilizers and pesticides in the soils. Biosurfactants have also been used in formulating poorly soluble organophosphorus pesticides.

2.2.3. In the food industry

In the food industry, biosurfactants are used as emulsifiers for the processing of raw materials. Emulsification plays an important role in forming the right consistency and texture as well as in phase dispersion. Other applications of surface-active compounds are in bakery and meat products, and in the emulsification of partially broken fat tissue. Lecithin and its derivatives are currently in use as emulsifiers in food industries worldwide.

2.2.4. Biosurfactants and phytoremediation

Heavy metals are the main culprit among the inorganic pollutants. At higher concentration of all the metals are toxic as they form the free radicals and cause oxidative stress. Also they disturb the normal activity of some essential enzymes and pigments by replacing them. But by using both metal resistant and biosurfactant producing bacteria, the capability of the plant can be increased for phytoremediation. For example biosurfactant producing *Bacillus* sp. J119 strain can increase the efficiency of the plant growth of rape, sundagrass, tomato and maize and also uptake of cadmium (Singh et al., 2008). From this analysis it is clear that the species taken for this purpose has the root colonization activity. So a microbe-helping phytoremediation process is developed for the remediation of heavy metals.

2.2.5. In environment pollution control

It can be efficiently used in handling industrial emulsion, control of oil spill, biodegradation and detoxification of industrial effluents and bioremediation of contaminated soil.

2.2.5.1. In toxic metal remediation

Contamination of toxic metals is a great threat to the ecosystem. Even if a small concentration of toxic metal is very dangerous to the ecosystem for their toxicity. Remediation of the soil that is contaminated with heavy metal is possible by various

techniques (Asci et al., 2010). The biological methods are like using plants (phytoremediation) or microbes (bioremediation), metal can be removed from soil. Toxic metals can only transfer from one chemical form to another, toxic to non toxic as they are not degradable. Some microbes can metabolize the toxic metals and also accumulate them intracellularly. They can also change the mobility of the metals by producing various substances and by pH (Briuns et al., 2000; Ledin, 2000). Biosurfactants helps in the remediation of toxic metals from soil by making complexes with the metals. They create a non-ionic form with the metals by ionic bonds which is very stronger than the bonds formed by the soil and metals. By lowering the interfacial tensions, the metals are adsorbed in the soil. Also by biosurfactant micelles, the metal ions can be removed as the polar head present in peripheral region of the micelles and it has the potential to mobilize the metal ions in the water (Mulligan and Gibbs, 2004; Singh and Cameotra, 2004).

2.2.5.2. In oil recovery

In microbial enhanced oil recovery (MEOR), to lower the interfacial tension at the oil–rock interface, microorganisms in reservoir are stimulated to produce polymers and surfactants which aid MEOR. In the reservoir microorganisms are usually provided with low-cost substrates, to produce MS (microbial surfactants) *in situ.* Such low cost substrates are molasses and inorganic nutrients, used to promote growth and surfactant production. In oil reservoirs under extreme conditions such as high temperature, pressure, salinity, and low oxygen level the microorganisms must be able to grow, that is useful of MEOR in situ. Several aerobic and anaerobic thermophiles tolerant of pressure and moderate salinity have been isolated which are able to mobilize crude oil in the laboratory. About 27% of oil reservoirs in USA are amenable to microbial growth and MEOR. Significant increase in oil recovery was reported in some field studies carried out in US, Czechoslovakia, Romania, USSR, Hungary, Poland, and The Netherlands. (Karanth et al., 1999).

2.2.5.3. In Hydrocarbon degradation

Hydroacrbon (C_XH_Y) degradation depends on the species which produce the substance that degrade the hydrocarbon. *Cladosporium resinae* produces fatty acids and phospholipids extracellularly which enhance the alkane degradation. PAHs are a class of compounds found throughout the environment in the air, soil and water with known harmful effects on humans and wildlife. These compounds are widely distributed and can be produced by natural or anthropogenic sources. Natural sources of PAHs include events such as volcanic releases and forest fires. Anthropogenic sources include incomplete fossil fuel combustion, industries, internal combustion and diesel engine exhausts, aviation exhaust, and cigarette

smoke. Although cigarette smoke accounts for the highest exposure threat to humans, vehicle exhaust is the main source of PAHs in the environment.

They are very hydrophobic organic compounds and are relatively insoluble in water. They have a high affinity for organic matter and when present in soil or sediments, tend to remain bound to solid particles and dissolve slowly in water. Poor solubility of PAHs is the major problem in biodegradation processes leading to the low bioavailability of PAHs as a substrate.

Biosurfactants are able to increase hydrophobic substrate solubility and provide a less aggressive environment for bacterial cells. Naturally, many microbes are able to degrade PAHs and can produce amphiphatic compounds similar to synthetic surfactants that can form micellar systems. When oil spill occur in aquatic environment, the polar hydrocarbon components dissolve in water (while lighter hydrocarbon components volatilize) and remain on the water surface. In aquatic environment, some microorganisms exhibit the emulsifying activity.

2.2.5.4. PAHs Biodegradation by microbes

According to Lawniczak and co-workers (2013), on the basis of some papers, they reviewed the biodegradation steps for biosurfactant based bioremediation strategy. For an efficient biodegradation strategy, bio-compatibility should be there in between the microbes or biosurfactants and pollutants. The native micro flora and the environmental factors should also be taken on to consideration in in-situ study. Biosurfactants can enhance hydrocarbon bioremediation by two mechanisms (Fig. 5).

1) Increase the substrate bioavailability for microorganisms
2) Interact with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells.

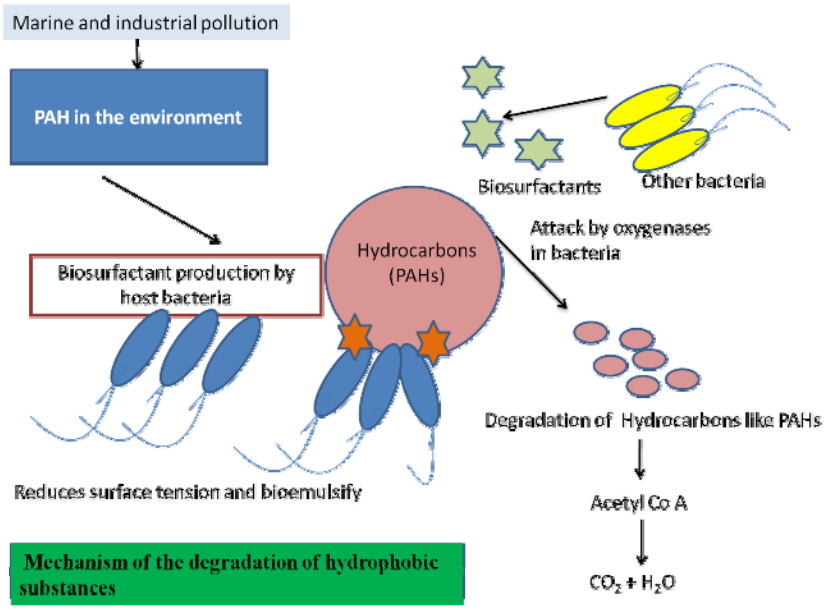


Fig. 5: The mechanism of Degradation of Polycyclic aromatic hydrocarbons (PAHs)

Naphthalene degradation by biosurfacatnt producing bacteria

Naphthalene and phenanthrene are degraded by *Pseudomonas alcaligenes* PA-10. This strain uses these PAHs as carbon and energy sources and also co-metabolizes the fluoranthene (Gordon and Dobson, 2001). According to Nnamchi et al. (2006), they isolated twenty four bacterial strains. They can uptake the naphthalene as a carbon and energy source for their growth. These strains were identified as the genus of *Pseudomonas, Burkholderia* or *Actinomycetes.* Among these strains, two strains *Pseudomonas aeruginosa* and *Burkholderia cepacia* degraded the anthracene and carbazole. During screening they also show the highest growth at OD_{600}.

Phenanthrene degradation by biosurfacatnt producing bacteria

According to Burd and Ward (1996), an extracellular surface-active substance with high molecular weight is secreted from a strain of *Pseudomonas marginalis.* This is composed of protein and lipopolysaccharide and promotes the growth of the strain on PAHs. According to Garcia-Junco et al., (2001) *Pseudomonas aeruginosa* 19SJ degrade the phenanthrene by producing rhamnolipid which increase the bioavailability of phenanthrene.

Here fifty strains of Pseudomonas (ARP) were isolated from crude oil polluted soil to test the degradation potential of phenanthrene. They used modified M9 minimal media (MM9) for enriched culture. ARP 26 and ARP 28 were selected for degradation as they showed the good result in MM9 agarised medium. They used the high performance liquid chromatography

(HPLC) for the determination of biodegradation rate of these two strains. ARP 26 showed 93 % degradation efficiency where as ARP 28 showed 98 %. Here they showed the degradation ability is due to plasmid and the plasmid pARP1 was estimated about 26 kb (Coral and Karagoz, 2005).

According to Arulazhagan and co-workers (2010), *Ochrobactrum* sp. (EU722312), *Enterobacter cloacae* (EU722313) and *Stenotrophomonas maltophilia* (EU722314), have showed the degradation of fluorene and Phenanthrene potently. They have taken different concentrations of NaCl like 30 g/l and 60 g/l and they have observed that the growth and degradation in 30g/l NaCl (95 %) is higher than the 60 g/l (39-45 %). They have taken the concentrations of PAHs are 5, 10, 20, 50 and 100 ppm. They found that at 30 g/l NaCl concentration, these strains showed good degradation and also by utilizing the yeast extract and these strains also showed the degradation ability of PAHs.

Some other PAHs degradation by microbes

In this paper the effect of Tween 80 and JBR was studied on flouranthene degradation by using the bacterial strain Pseudomonad. These two surfactants enhanced the degradation rate of fluoranthene by helping the strain *Pseudomonad alcaligenes* PA-10 as good growth factor. After 28 days it was monitored that the fluoranthene was removed with a significant amounts (45± 5 %) by comparing with an uninoculated control. This addition of biosurfactant increased the degradation rate of the phenanthrene (Hickey et al., 2007).

According to Ceyhan (2011), *Proteus vulgaris* has the potential to degrade the pyrene at a rate of 71.5% within 7 days (initial concentration is 0.5mg/ml). Also the metabolites that are produced during the pyrene degradation were detected by using TLC analysis and HPLC and these are detected as non-toxic and not persistent. Pyrene is a high molecular weight PAHs that contains four benzene rings (fused). But *P. vulgaris* has the effective and quick degradation property of pyrene. So this strain can be used in pyrene polluted area for bioremediation. It is the first pyrene degrading bacteria that is reported here.

In this paper studied, it is mentioned that *Mycobacterium* sp*., Corynebacterium* sp*., Nocardia* sp*., Pseudomonas* sp*., Rhodococcus* sp. and *Micrococcus* sp. were potentially capable of degrading pyrene hydrocarbon. At optical density OD_{600}, they showed highest growth rate. *Mycobacterium* sp. and *Corynebacterium* sp. can be used for pyrene polluted area as they showed a good degradation rate of pyrene like 89.1% and 79.4% (Kafilzadeh et al., 2012).

According to Sponza and Gok (2011), hydrocarbon rich petroleum sewage was degraded by a microbe in association with chlorophenols. The micelles of the biosurfactant

take this phenol molecule. So the toxicity of phenol based molecules was reduced due to the interaction between micelles of biosurfactant and these molecules. The microbes get nutrients and energy for growth as well as this enhanced the biodegradation of PAHs in the petroleum sewage. By some further studies, it was cleared that in petrochemical industry wastewater, by addition of rhamnolipid can also improve the biodegradation rate.

3. OBJECTIVES

On the basis of the above reviews, the following objectives of the present research work are taken in to consideration.

- Screening, Phenotypic and Biochemical characterization of biosurfactant producing marine bacteria.
- Growth optimization of the isolates in different Carbon and Nitrogen sources.
- Extraction and Chemical Characterization of biosurfactant produced from the isolates.
- Antioxidant, anti adhesive and antimicrobial activity study of the extracted biosurfactant.
- Biosurfactant based bioremediation of PAHs (polycyclic aromatic hydrocarbon).

4. MATERIALS AND METHODS

Fourteen bacterial strains were isolated earlier from Paradeep port, Vishakhapatnam, Rishikulya, Bhitarakanika marine water and streaked in Luria Bertani agar plates for several times and maintained at pure culture.

4.1. Colony Morphology

Colony morphology of the isolated bacterial colonies was observed for twenty four hours incubated cultures.

4.2. Screening for Biosurfactant activity

4.2.1. Drop collapsing test

Fourteen bacterial strains were grown overnight in Luria Bertani broth with 10 mM $MgCl_2$. Next day these were sub-cultured in to MMMF media and incubated for 24 h at 23°C. 10 µl of the supernatant of each strain was spotted on the polystyrene coated glass plate that was coated by immersion oil (Taguchi et al., 2006).

4.2.2. Oil spread method

Bacterial strains were inoculated in Luria Bertani broth with 10 mM $MgCl_2$ for 24 h. Next day 20 µl of immersion oil was layered uniformly to a 20 ml of distilled water that was kept in the petriplate. 20 µl of the culture was added to different spot on the immersion oil after doing vertex for 2 minutes. Then after 30 sec it was observed whether they are giving a clear zone or not. If a clear zone was found then it should be positive (Morikawa et al., 2000).

4.2.3. Emulsification test

The bacterial strains were inoculated in Luria Bertani broth with 10 mM $MgCl_2$ for overnight. Then 2 ml of the bacterial culture was transferred to a test tube and n-octane (1 ml) was added in to it. The mixture was vortexed for 2 min and then kept for 24 h to see the emulsification result.

Emulsification index = (Emulsification height/ Total height) × 100 (Cooper et al., 1987).

4.2.4. Blood Haemolysis Test

On the blood agar plates, the isolated fresh colonies were streaked and kept in incubator for 24 hours at 37°C. Then the plates were observed for the presence of the clear zones around the colonies which will indicate the biosurfactant producing organisms (Satpute et al., 2008).

4.3. Physical characterization of bacterial isolates

4.3.1. Gram Staining

First clean and dry glasses slides were taken and then smears of bacterial suspensions were made with one drop of distilled water. These slides were heat fixed and the fixed smears were flooded with crystal violet solution and kept for 1 min and then these slides were rinsed of with distilled water. Then these slides were treated with Gram's iodine solution and allowed to remain for 1 min. Again these slides were rinsed with distilled water. Then these slides were treated with 1-2 drops of Gram's decolourizer and kept for 1-5 sec. Then the slides were rinsed with water. Lastly these were flooded with safranin and kept for 1 min, then again ringed with distilled water. These slides air dried and observed under light microscope at 40X objective. If the cells retained pink colour of safranin, then these were identified to be Gram negative and if they retained violet colour of crystal violet, they were identified as Gram positive.

4.3.2. Characterization of biosurfactant producing bacteria by Scanning Electron Microscopy (SEM)

The freshly cultured strains were centrifuged at 8000 g, 4°C for 5 min and the cells were washed with 0.1 mM phosphate buffer saline (PBS) for 3 times. Then the cells were fixed by adding 2 % glutaraldehyde prepared in 0.1 M PBS and incubate at room temperature overnight for fixation. Then next day the cells were washed thrice with PBS. Then these cells were centrifuged at 8000 g, 4˚C for 5 min. Then de-hydrolysis was done of each sample by different ethanol concentrations like 30 %, 50 %, 70 %, 90 %, 100 % and incubate for 18 hours. Then these were incubated for 1 h with ethanol (100 %). Then these slides were air dried and observed at various resolutions under SEM.

4.4. Biochemical Tests of bacterial isolates

4.4.1. Mannitol Motility Test

Freshly cultured bacterial strains were inoculated in mannitol motility nitrate agar by a straight needle and incubated for 24 h. Then after incubation these strains were checked for motility. The strains are non motile if they showed growth along the line of the inoculation and if they showed growth by spreading over the medium then they are motile. Also by this mannitol motility test bacterial strains were tested whether they can ferment mannitol or not. If the colour of the medium changes from red to yellow then it they will give positive result.

4.4.2. Nitrate reduction test

In this test 1-2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl-Napthylamine reagent were added to the kit medium to test whether these bacterial strains are able to convert nitrate to nitrite or not. If the colour immediately changed in to pinkish red colour on addition of reagent indicates positive reaction and if there is no change in colour it shows negative result.

4.4.3 Sulphide Indole Motility (SIM) Test

14 strains were inoculated in Sulphide indole motility (SIM) media and incubated these to test whether they are motile or non motile and whether they are producing sulphide or not. If the colour of the medium will change from yellow to black then H_2S production result will be positive.

4.5. Growth Curve

Bacterial strains were inoculated in Luria Bertani broth and 300 µl of the freshly inoculated bacterial cultures were taken in micro-titre plate (Tarson). Then O.D. was measured at 595 nm in the ELISA Reader (Perkin Elmer). Then optical densities of each strain were regularly taken at every 2 h interval for 24 h and these were plotted against time to determine the growth curve.

4.6. Growth optimization of the strains in different carbon and Nitrogen sources

4.6.1. Growth optimization in Carbon sources

Two aliphatic carbon sources - Glycerol and Sucrose and five aromatic carbon sources - Kerosene, Pyrene, Biphenyl, Naphthalene, Phenanthrene were taken to optimize the growth of these strains. 2 % of each aliphatic carbon sources were added to Bushnell Haas Broth (BHB) with supplementation of NaCl (19.450 mg/l) and concentration of 100 mg/l of each aromatic source were taken and the absorbances were monitored at 595 nm (Onwosi and Odibo, 2012).

4.6.2. Growth optimization in Nitrogen sources

Three nitrogen sources - KNO_3, Urea and Yeast extract were taken and 2 % of each was added to the BHB (supplementation with NaCl- 19.450 gm/l) with the respective optimised carbon sources for the growth optimization of these strains. Absorbance was taken at 595 nm (Onwosi and Odibo, 2012).

4.7. Extraction of Biosurfactant

The five strains were inoculated in Bushnell Haas Broth with supplementation with NaCl (19.450 gm/ l) with respective carbon and nitrogen sources and then incubated at 25°C for 7

days with shaking conditions. After incubation supernatants were collected by centrifuging at 6000 rpm, 4°C for 20 minutes. Then pH of these supernatants was adjusted by adding 1M $H2SO_4$. Then equal volume of chloroform: methanol was added to these supernatant in the ratio of 2:1. These are shaken well for proper mixing and then left overnight for evaporation. If white coloured precipitates were seen in the interface between the two liquid then the presence of biosurfactant is confirmed (Dhouha et al., 2012).

4.8. Characterization of Biosurfactant

4.8.1. Carbohydrate and protein estimation

By phenol-sulfuric acid method (Dubois et al., 1956) carbohydrate estimation of the biosurfactant was done and by Bradford method (Bradford, 1976) protein estimation was done.

4.8.2. Surface tension Measurement

50 ml of the crude biosurfactant of 5 strains were taken for surface tension measurement with respect to distilled water. By digital tensiometer the surface tensions of each strain were plotted. Hence the surface tensions with respect to distilled water were determined (ABU-Ruwaida et al., 1991).

4.8.3. Fourier Transform Infrared analysis (FTIR)

FTIR was used to determine the chemical nature of the biosurfactants

FTIR spectroscopy was carried out using crude biosurfactant extract obtained from the acid precipitation of the cell free culture supernatant. IR Prestige- 21Fourier Transform Infrared spectrophotometer (Samadzku, Japan) was used to determine the chemical nature of the biosufactant by the KBr pellet method (Das et al., 2008a, b; Mukherjee et al., 2009).

4.9. Antimicrobial activity test

20 ml Muller Hinton Agar media with supplementation of NaCl (19.450gm/l) was prepared each for petriplates on each of which 3 wells were made and were named as A, B and C respectively. The plates were swabbed with *Bacillus, Shigella, Streptococcus, Escherichia coli, Proteus, Salmonella.* To the wells A, purified biosurfactants were added, to the wells B, diluted biosurfactants (10 fold) were added and to the wells C, distilled water (control) was added. The plates were kept in incubation at 37°C for 24 hours. The presence of clear zone marked the antimicrobial activity of biosurfactant. Three readings of the clear zone diameter were taken for each well and the mean was calculated to determine the actual zone diameter (Rodrigues et al., 2006).

4.10. Antioxidant activity test

The antioxidant potential of the biosurfactant was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH method is widely used and the easiest method to determine the antioxidant activity of compounds. The aliquots of the different concentrations of the biosurfactant were added to 5.0 ml of a 0.004% (w/v) solution of DPPH. Absorbance at 517 nm was determined after 30 min, and IC50 (the half maximal inhibitory concentration) was determined. IC50 value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals. The radical scavenging activity at different biosurfactant concentration was calculated by the

Equation: $\mathbf{S_{DPPH} = 100\ x\ (1 - A_{sample}/A_{DPPH})}$

Where A_{sample} indicates the absorbance of the solution in the presence of test samples, and A_{DPPH} indicates the absorbance of the DPPH solution in the absence of the test samples (Yalcin and Cavusoglu, 2010).

4.11. Anti adhesive test

200 µl of the crude biosurfactants (100 mg/ ml in PBS) were filled in the wells and the control wells were filled only with PBS. The plate was then incubated at 4˚C for 18 h. After 18 h the plate was washed with PBS for 3 times. The pathogenic bacteria cultures *Streptococcus pneumoniae* and *Bacillus* sp. were centrifuged and pellets were collected. These pellets were resuspended in PBS and then added to these wells. The plate was then kept in incubator at 4˚C for 4 h. The plate was washed with PBS for 3 times. Then 200 µl methanol was added and fixed for 15 min. The plate was dried and then stained with 2% crystal violet (200 µl) for 5 min. The dye was re-solubilized with 200 µl of 33% (v/v) glacial acetic acid per well. Then absorbance was measured at 595 nm.

$$\textbf{\% of microbial inhibition} = [1-(A_c/A_0)] \times 100$$

Where A_C = Absorbance of the well with a biosurfactant concentration c.

A_0 = Absorbance of the control well (Rufino et al., 2011).

4.12. Biodegradation of Polycyclic aromatic Hydrocarbon (PAH) by Biosurfactant

4.12.1. Phenanthrene Biodegradation

First bacterial cultures were inoculated in Luria Bertani broth for 24 h. Next day 100 µl of these bacterial cultures were sub cultured in 50 ml Bushnell Haas media (with supplementation of NaCl 19.450 gm/l) with 100 mg/l of phenanthrene for 7 days for enrichment culture. At 7th day the pellets of each bacterial culture was collected by centrifuging at 6000 rpm, 10 min at 4ºC. Then these pellets were re-suspended in Bushnell

Haas broth (2 ml). Then the O.D. of each bacterial pellet (300 µl) was taken at 595 nm in ELISA Plate Reader. As O.D. of each strain was found less than 1, then 50 µl of enriched pellet of each strain was transferred to 5 ml of Bushnell Haas broth with Phenanthrene (100 mg/l) and kept in shaker incubator (in dark) at 180 rpm, 37˚C. Then at Day-1, Day-3, Day-5, Day-7, extraction was done by adding the equal volume of n-Hexane. After adding n-Hexane, it was vortexed for 5 minutes and then centrifuged at 6000 rpm for 10 min at 4°C to collect the Hexane layer. Then O.D. of the Hexane extract was taken at 292 nm and also scanned from 200 nm to 400 nm (Tao et al., 2007).

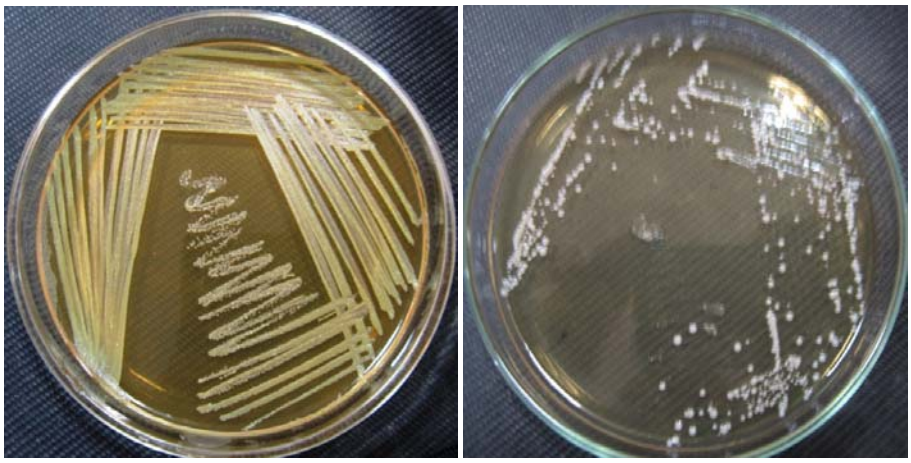
4.12.2. Naphthalene Biodegradation

First bacterial cultures were inoculated in Luria Bertani broth for 24 h. Next day 100 µl of these bacterial cultures were sub cultured in 50 ml Bushnell Haas media (with supplementation of NaCl 19.450 gm/l) with 100 mg/l of naphthalene for 7 days for enrichment culture. At 7th day the pellets of each bacterial culture was collected by centrifuging at 6000 rpm, 10 min at 4˚C. Then these pellets were re-suspened in Bushnell Haas broth (2 ml). Then the O.D. of each bacterial pellet (300 µl) was taken at 595 nm in ELISA Plate Reader. As O.D. of each strain was found less than 1, then 50 µl of enriched pellet of each strain was transferred to 5 ml of Bushnell Haas broth (with NaCl 19.450 gm/l) with Naphthalene (100 mg/l) and kept in shaker incubator (in dark) at 180 rpm, 37°C. Then at Day-1, Day-3, extraction was done by adding the equal volume of n-hexane. After adding n-Hexane, it was vortexed for 5 min and then centrifuged at 6000 rpm for 10 minutes at 4°C to collect the Hexane layer. Then O.D. of the Hexane extract was taken at 254 nm and also scanned from 200 nm to 400 nm (Tao et al., 2007).

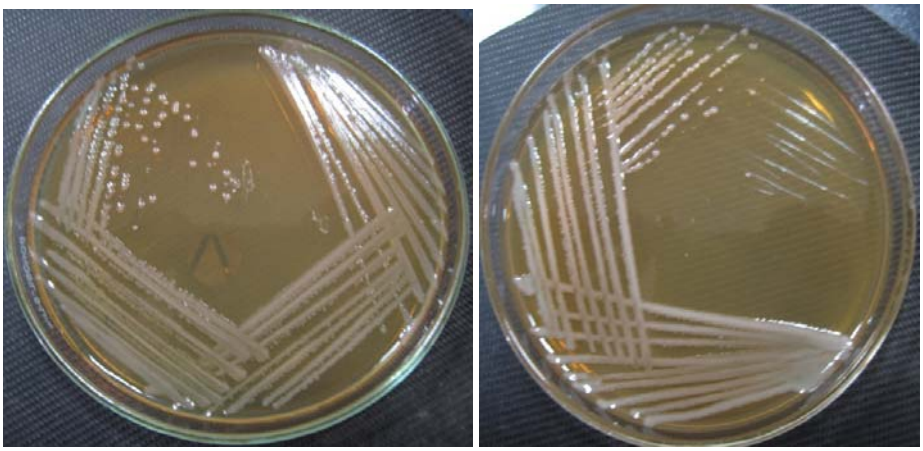
5. RESULTS

5.1. Cell Morphology

Cell morphologies of these five strains- JV502, JV501, JP022, JV201 and JP011were given below (Fig. 6).



a) JV502 b) JV501



c) JP022 d) JV201

e) JP011

Fig. 6: Cell morphology of the isolated strains.

5.2. Screening for biosurfactant activity

5.2.1. Drop collapse test

10 µl cell suspension of each strain was placed on the polystyrene coated glass plate that was coated by immersion oil. If the cell suspension contains biosurfactant then the drop collapses or spread due to the reduction of hydrophobic surface and if there is no biosurfactant in the cell suspension then the drops remain stable as the polar water molecules are repelled from the hydrophobic surface. Stability of the drop depends on biosurfactant concentration. Only two strains JV801 and NR802 gave negative results and rest gave positive results. These were given below (Table 4).

Table 4: Results of Drop Collapse test

Strain Name	Results
JV501	positive
JV201	positive
JV502	positive
JP022	positive
JV202	positive
JV101	positive
JP011	positive
JV801	negative
NE3B02	positive
NE3B01	positive
NP202	positive
NP103	positive
ATCC	positive
NR802	negative

5.2.2. Oil spread method

Cell free culture broth of 14 strains were taken and added to the plate that contained distilled water and oil. The 12 strains JV501, JV201, JV502, JP022, JP011, NE3B02, NE3B01, NP202, NP103, ATCC, NR802 and JV801showed the zone of displacement in oil.

The zone of displacement showed the biosurfactant production in these strains and the results were noted down (Table 5).

Table 5: Results of Oil spread method

Strain Name	Results
JV501	positive
JV201	positive
JV502	positive
JP022	positive
JV202	negative
JV101	negative
JP011	positive
JV801	positive
NE3B02	positive
NE3B01	positive
NP202	positive
NP103	positive
ATCC	positive
NR802	positive

5.2.3. Emulsification test

In emulsification test, JP022 showed 41% of emulsification activity and the other strains ranging between 35-40 % of emulsification activity and negative emulsification activity was observed in NP202, NP103 and JV101 (Fig. 7).

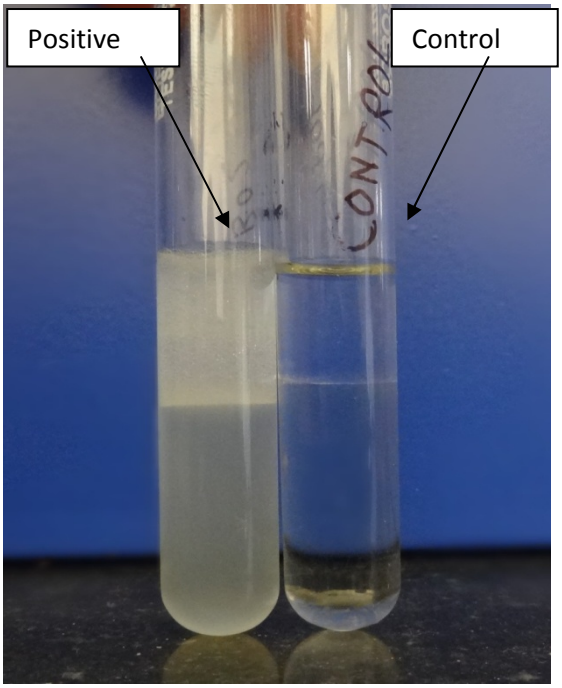
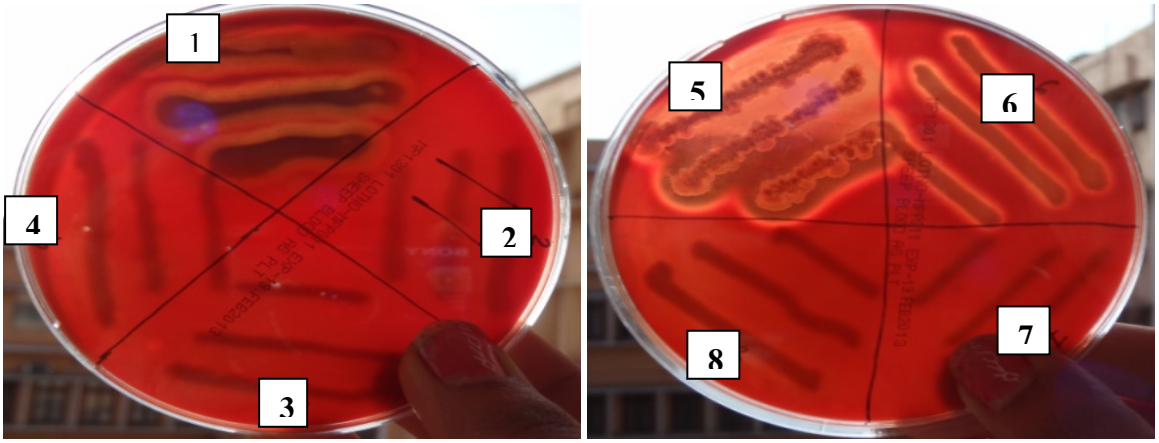


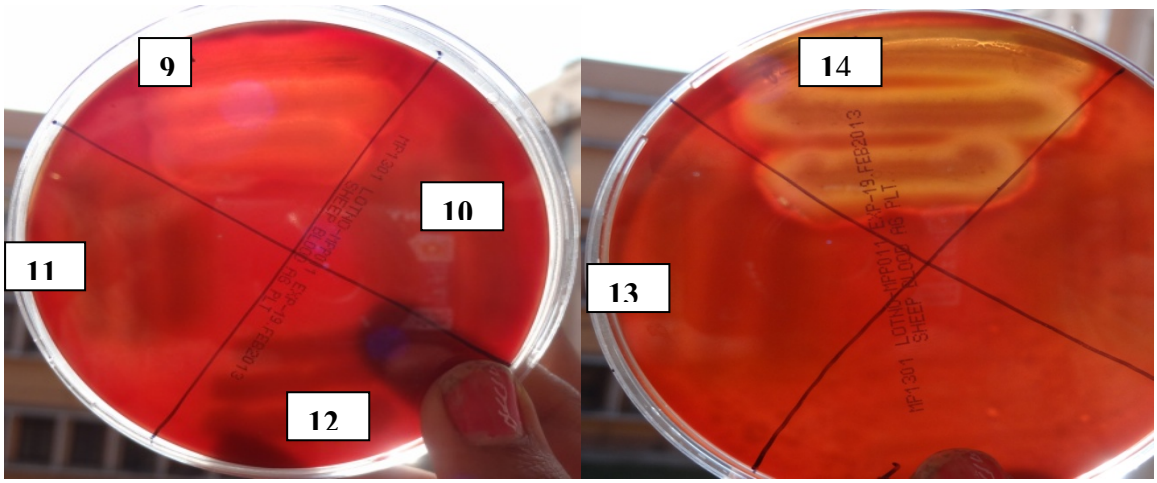
Fig. 7: The tubes showing Emulsification activity

5.2.4. Blood Haemolysis Test

14 strains were streaked on blood agar plates. Among them JV501, JV201, JP022, JP011 and ATCC showed haemolytic activity by forming a clear zone around the colonies (Fig. 8).



(a) 1-JP011, 2-JV502, 3-JV101, 4-JV202 (b) 5-JP022, 6-JV201, 7-NE3B01, 8-NE3B02



(c) 9-ATCC, 10-NR802, 11-NP103, 12-NP202 (d) 13-JV801, 14-JV501

Fig. 8: Haemolytic activity of the isolates, JP011- α haemolysis, JP022- β haemolysis, JV201- β haemolysis, ATCC- haemolysis, JV501- β haemolysis and others are showing negative result)

Among these fourteen strains, five strains were giving good results in screening of biosurfacatant test, so these five strains were taken for further study.

5.3. Physical characterization of bacterial isolates

5.3.1. Gram staining

Cell morphology of these 10 strains were studied by gram staining and observed under oil immersion microscope. Among these strains, JV501 was Gram positive bacteria and others were Gram negative. Similarly JV501 was coccus and others were rods. The results have been given below in the Table 6.

Table 6: Cell morphology of the isolates

Strain Name	Colour	Gram staining	Shape
JV501	Purple	+ ve	Cocci
JV201	Pink	-ve	Rods
JV502	Pink	-ve	Rods
JP022	Pink	-ve	Rods
JP011	Pink	-ve	Rods

5.3.2. Characterization of biosurfactant producing bacteria by Scanning Electron Microscopy (SEM)

Cell surface topography of these five strains was observed by SEM image (Fig. 9).

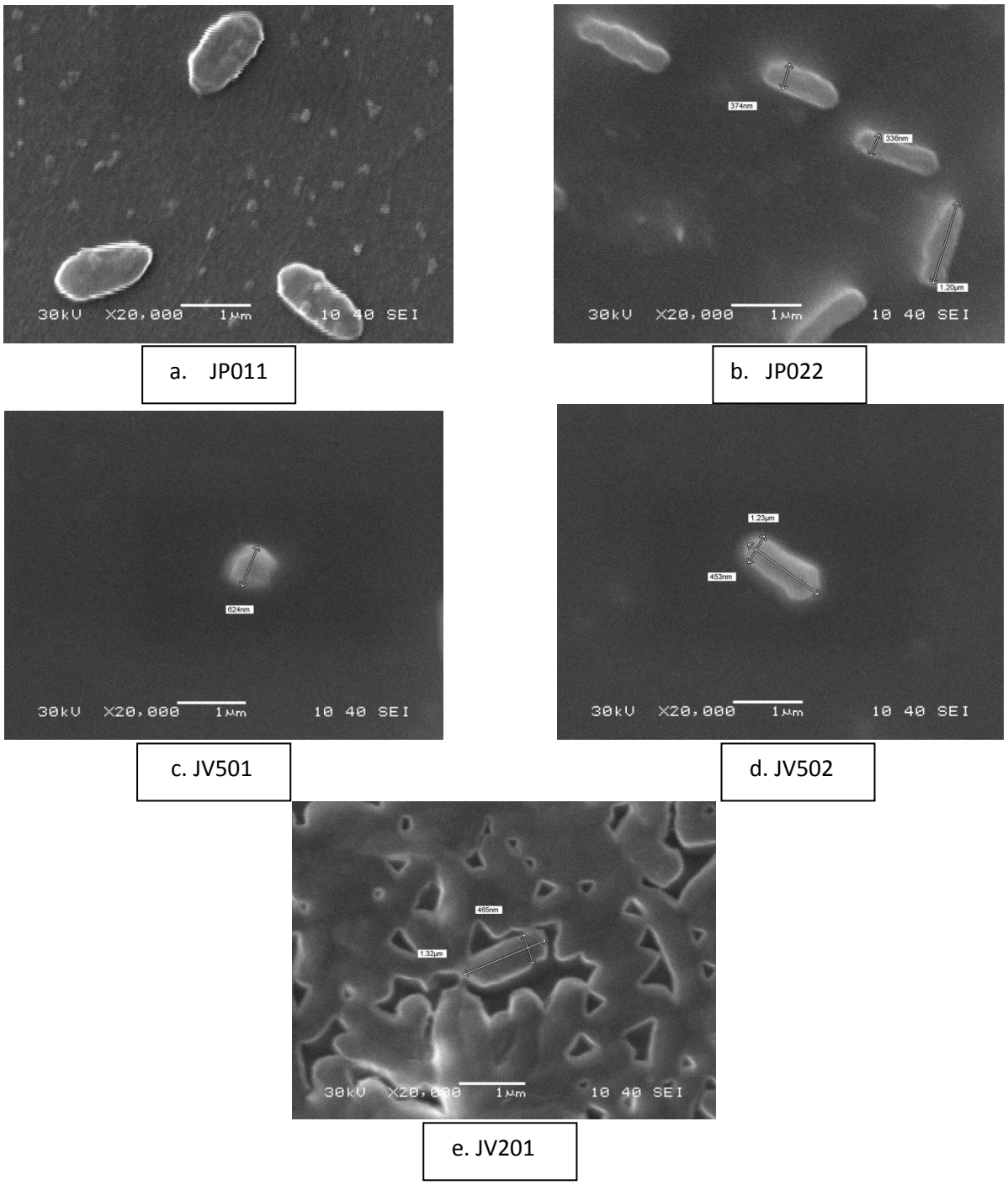


Fig. 9: SEM results of the isolates

5.4. Biochemical tests

The results of all biochemical tests like Mannitol motility test, Nitrate reduction test, Sulphide indole motility test are done (Table 7).

5.4.1. Mannitol Motility Test

In this test, JV501, JV201, JV502 were showing positive result and others were showing negative result (Fig. 10). In case of motility test, all strains were found motile excluding JP022 and JP011.

5.4.2. Nitrate Reduction Test

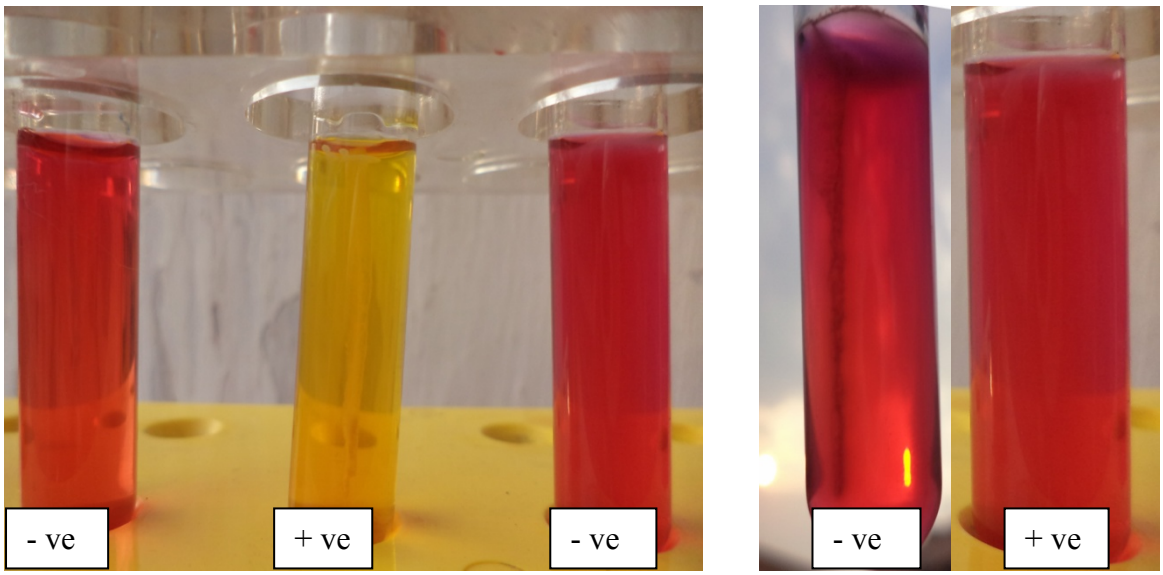
Only JV501, JV502 were showing positive result and others were showing negative result.

5.4.3. Sulfide indole motility (SIM) test

All strains showed negative result.

Table 7: Results of all biochemical tests

Strain name	Mannitol test	Motility test	Nitrate reduction test	Sulfide production test
JV501	+ ve	Motile	+ ve	- ve
JV201	+ ve	Motile	- ve	- ve
JV502	+ ve	Motile	+ ve	- ve
JP022	- ve	Non-motile	- ve	- ve
JP011	- ve	Non-motile	- ve	- ve



A) **B)**

Fig. 10: A) Mannitol test, + ve showing mannitol fermentation, B) Motility test, + ve showing Motile and – ve showing Non-motile

5.5. Growth Curve

Five strains were freshly inoculated to check their growth curve. In ELISA Reader (Perkin Elmer) the optical densities were measured and plotted against time (Fig. 11).

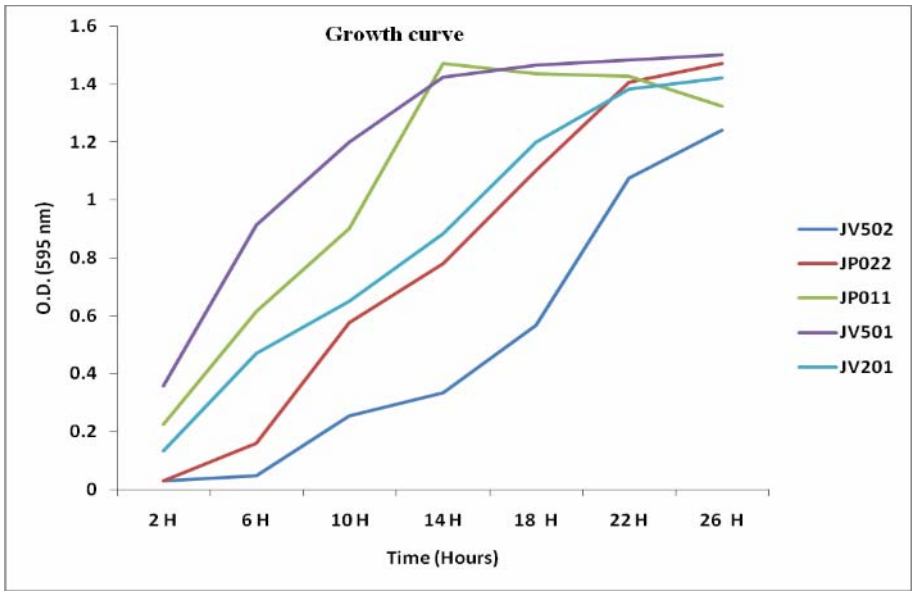


Fig. 11: Growth curve of the bacterial isolates

5.6. Growth optimization in different carbon and nitrogen sources

5.6.1. In different Carbon Sources

Two aliphatic carbon sources (Glycerol and Sucrose) were taken to monitor the growth of these strains.

Aliphatic Carbon sources

Strain JV502, JV201 and JP011 showed good growth in glycerol (Fig. 12).

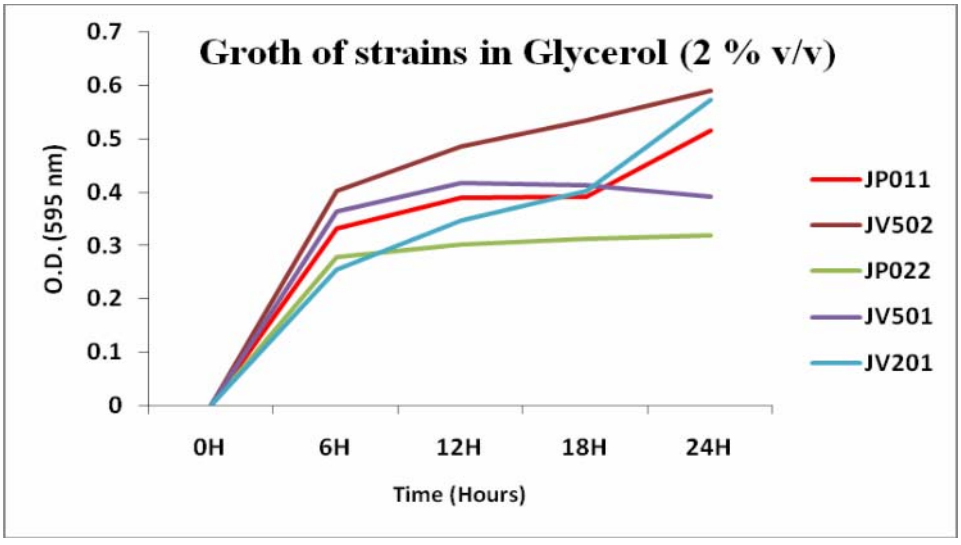


Fig. 12: Growth optimization of the isolates in Glycerol

Strain JP011, JV501, JV201 should good results in sucrose as carbon source (Fig. 13).

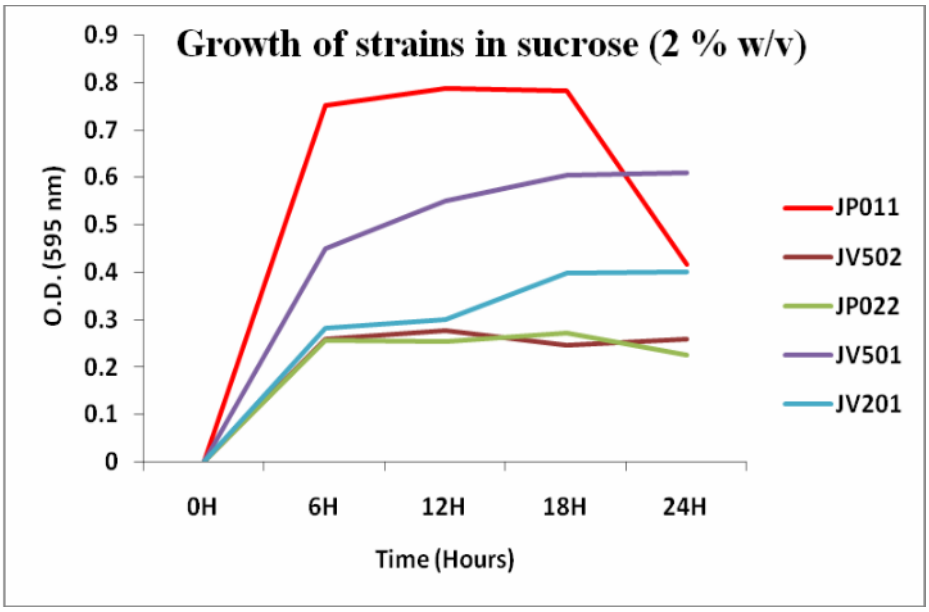


Fig. 13: Growth optimization of the isolates in Sucrose

Aromatic carbon sources

JV501, JV201 showing good growth results in Kerosene (Fig. 14). JV201, JV501, JV502 showed the good growth in Pyrene medium (Fig. 15). JV501, JP011 and JV502 showed good growth in Biphenyl (Fig. 16). In Naphthalene JV501, JV201 and JP022 showed good growth (Fig. 17). In Phenanthrene also JV501, JV201 and JP011 showed good result (Fig. 18).

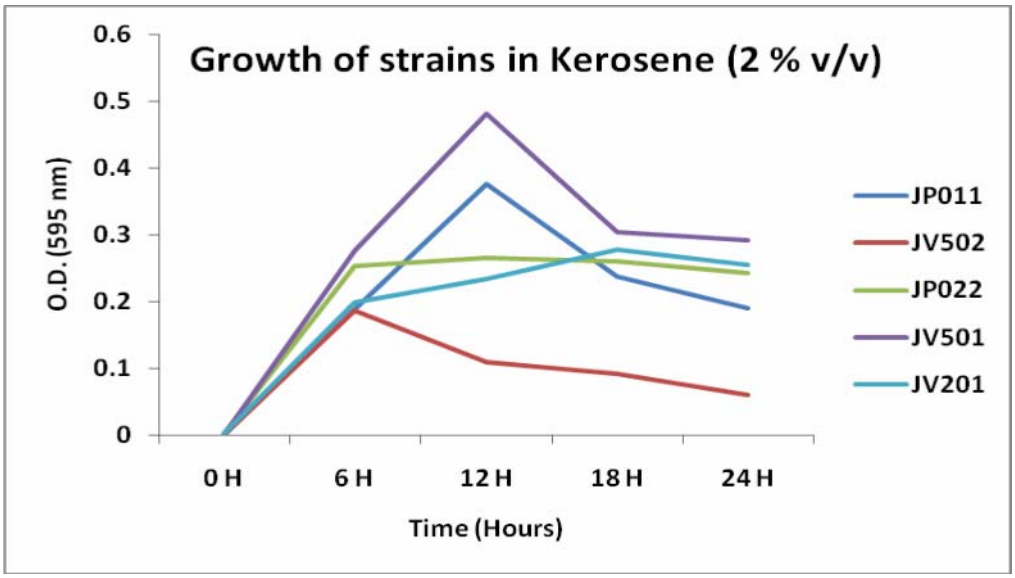


Fig. 14: Growth optimization of the isolates in Kerosene

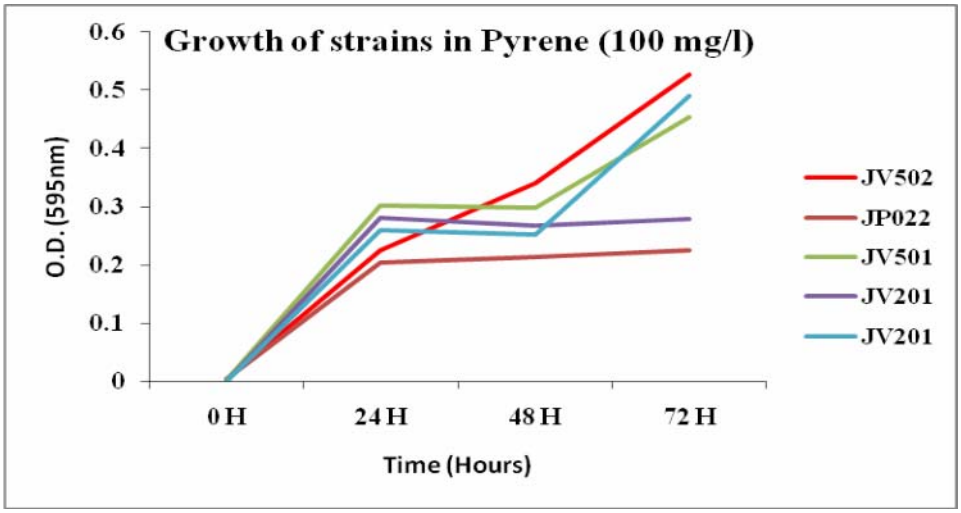


Fig. 15: Growth optimization of the isolates in Pyrene

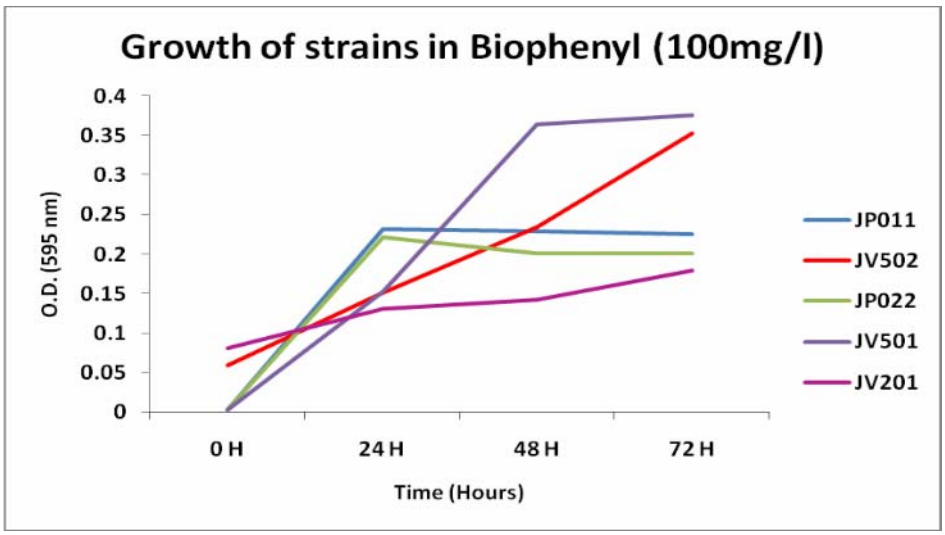


Fig. 16: Growth optimization of the isolates in Biphenyl

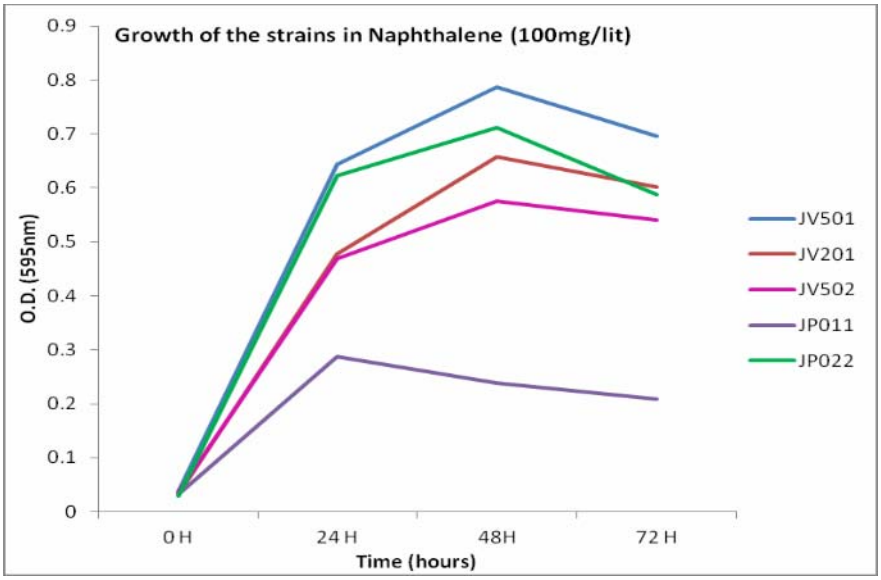


Fig. 17: Growth optimization of isolates in Naphthalene

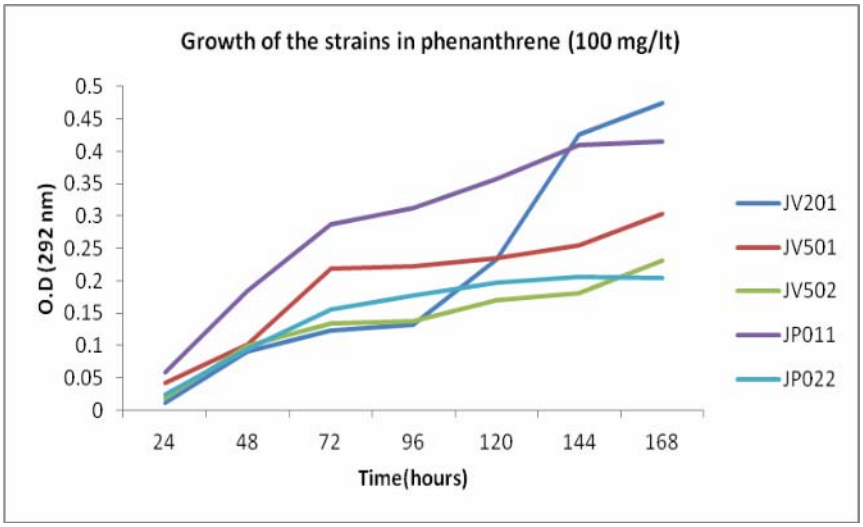


Fig. 18: Growth optimization of the isolates in Phenanthrene

5.6.2. Growth optimisation of the strains in different Nitrogen sources

The strains were optimised for better growths in three nitrogen sources yeast extract, urea, potassium nitrate. All the strains showed good growth in presence of yeast extracts (Fig. 19) whereas strain JV501, JV502 showed good growth in urea (Fig. 20) and also in Potassium nitrate (Fig. 21).

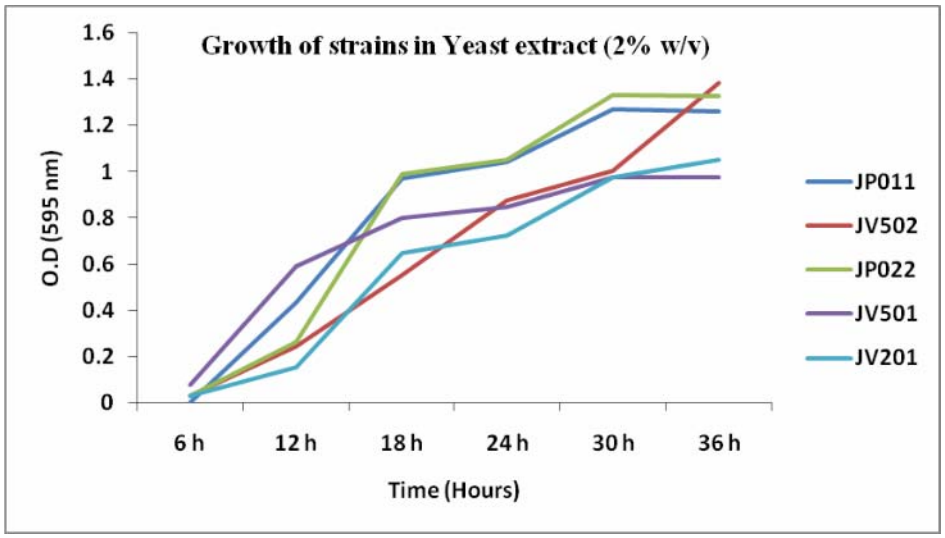


Fig. 19: Growth optimization of the isolates in Yeast Extract

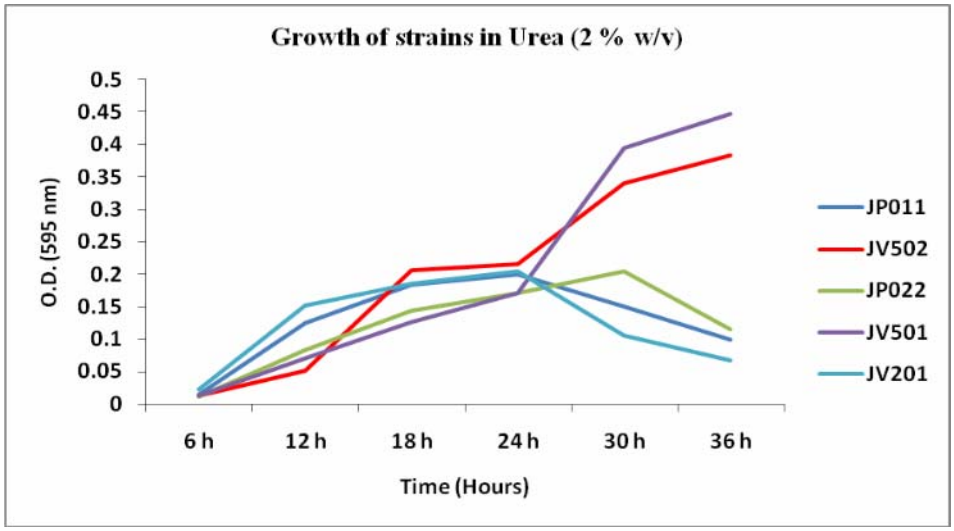


Fig. 20: Growth optimization of the isolates in Urea

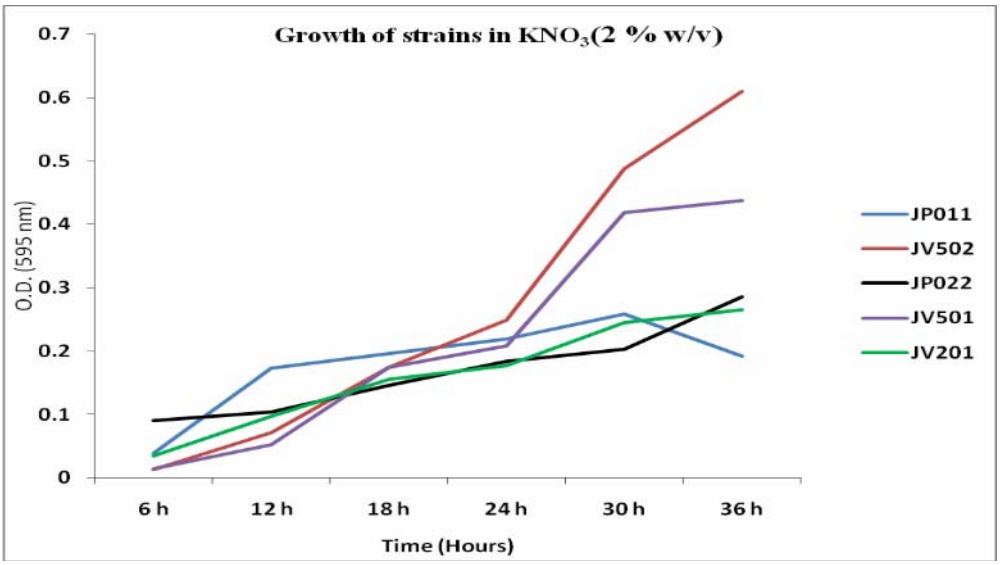


Fig. 21: Growth optimization of the isolates in Potassium Nitrate

5.7. Extraction of Biosurfactant

The five strains were inoculated in Bushnell Hass Broth with respective carbon and nitrogen sources for 7 days in which they had given good results. Supernatants were obtained by centrifuging at 6000 rpm at 4°C for 20 min and equal volume of chloroform: methanol (ratio 2:1) was added for acid precipitation with 1M H_2SO_4. After the pH was adjusted to 2, these were left overnight for evaporation and if white coloured precipitate were seen in between two immiscible liquids then biosurfactant production was observed. The biosurfactant productions of all five strains were observed (Fig. 22).

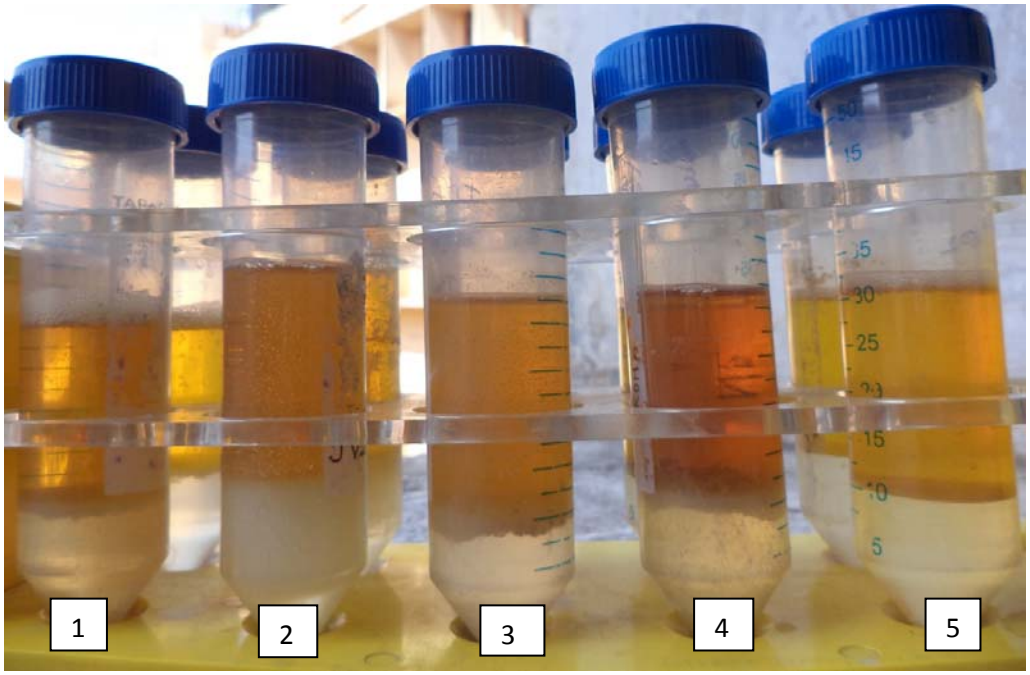


Fig. 22: Biosurfactant production (1-JV501 2-JV201, 3- JV502, 4-JP022, 5-JP011, All are producing approximately 100mg/25ml)

5.8. Characterization of Biosurfactant

5.8.1. Carbohydrate estimation

The concentration of carbohydrate present in the biosurfactants was calculated from the standard curve (Fig. 23). JV201 showed the maximum carbohydrate content (Table 8).

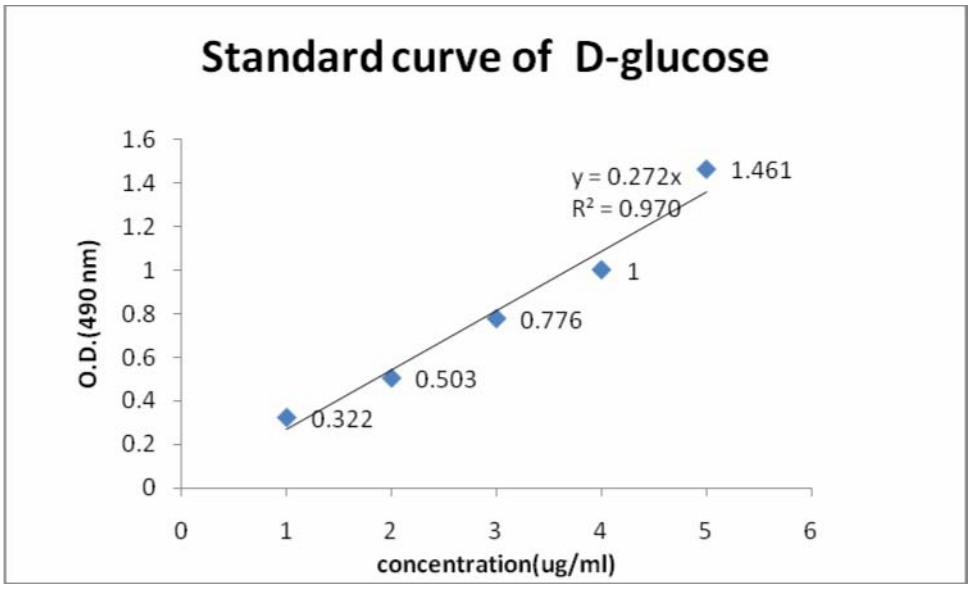


Fig. 23: Standard curve of D-glucose

Table 8: Carbohydrate estimation of the biosurfactants

Strain name	O.D. at 490 nm	Concentration(µg/ml)
JV501	0.035	0.217979
JV201	0.172	0.709865
JV502	0.063	0.318511
JP011	0.028	0.192847
JP022	0.048	0.264655

5.8.2. Protein Estimation

The concentration of protein present in the biosurfactants was calculated from the standard curve (Fig. 24). JV201 showed the maximum carbohydrate content (Table 9).

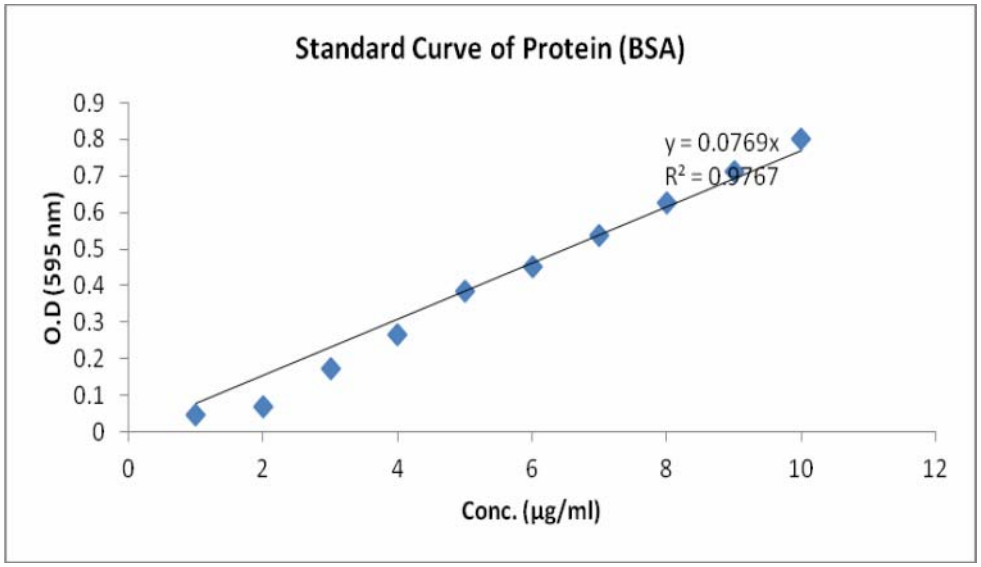


Fig. 24: Standard curve of Protein

Table 9: Protein Estimation of the biosurfactants

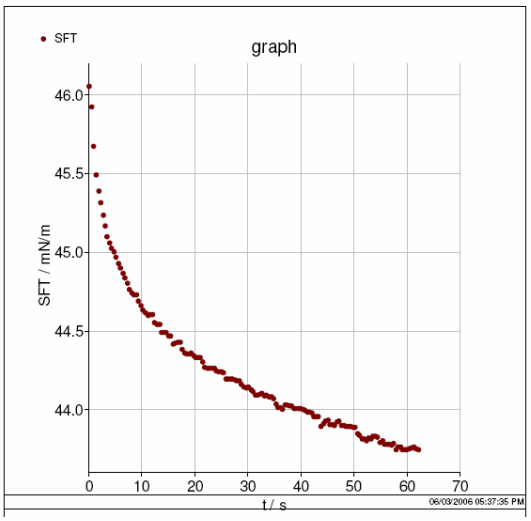
Strain name	O.D. at 595 nm	Concentration(µg/ml)
JV501	0.022	1.122537
JV201	0.183	2.952162
JV502	0.015	1.042988
JP011	0.017	1.065717
JP022	0.019	1.088445

5.8.3. Surface Tension Measurement

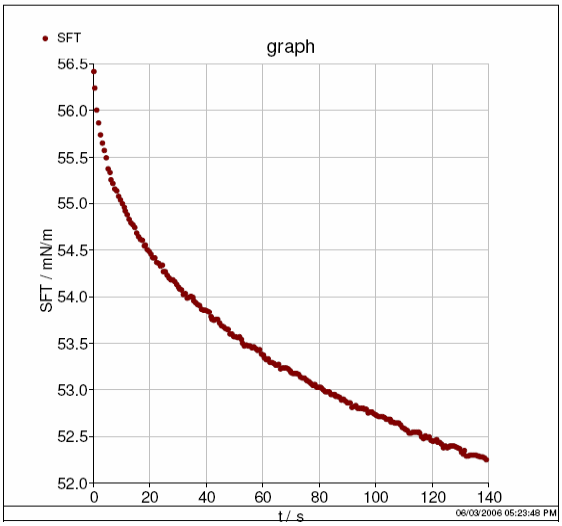
Surface tension of crude biosurfactant was determined by a digital Tensiometer with respect to distilled water (Table 10, Fig. 25).

Table 10: Surface tensions of the isolates

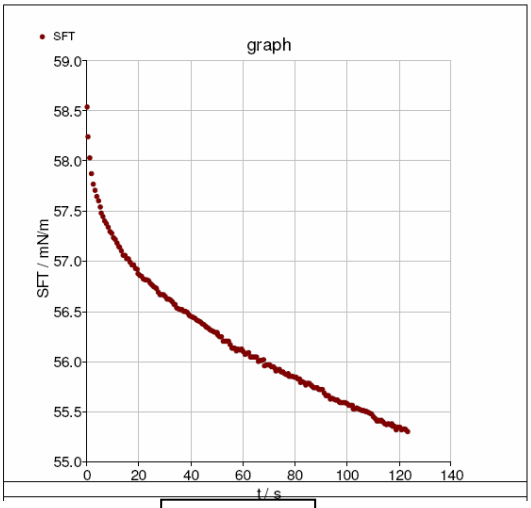
Strain name	Surface tension
JV501	55.234±0.028 mN / m
JV201	55.368 ± 0.028 mN / m
JV502	51.251 ± 0.028 mN / m
JP011	43.776 ± 0.029 mN / m
JP022	52.292 ± 0.028 mN / m



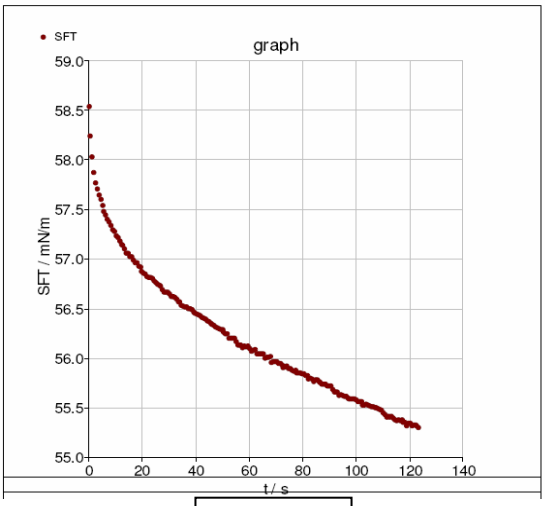
a. JP011



b. JP022



c. JV201



d. JV501

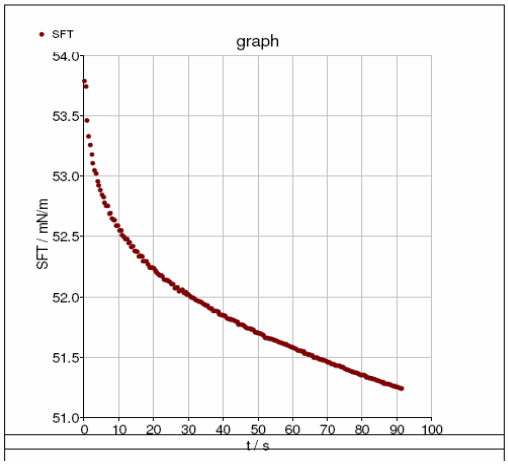


Fig. 25: Surface Tension Measurement of the isolates

5.8.4. Fourier Transform Infrared analysis (FTIR)

The determination of functional group present in the crude biosurfactant was determined with the help of Fourier Transform Infrared Spectroscopy. These are the FTIR results of 4 strains (Fig. 26, 27, 28, 29)

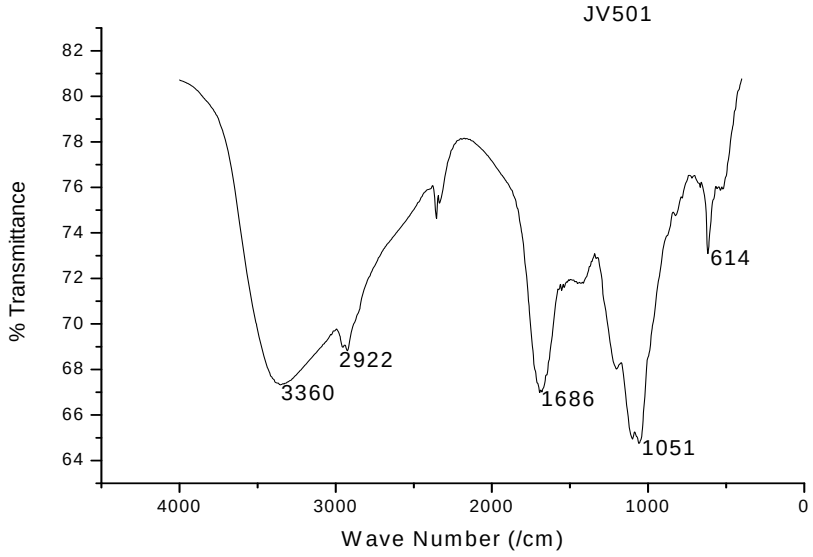


Fig. 26: FTIR result of JV501

Inference

Wave number	Bonds	Functional group
3360	N–H stretch	Primary, Secondary amines, Amides
2922	C–H stretch	Alkanes

1686	C=O stretch	Carbonyls (general)
1051	C–N stretch	Aliphatic amines
614	C–Cl stretch	Aalkyl halides

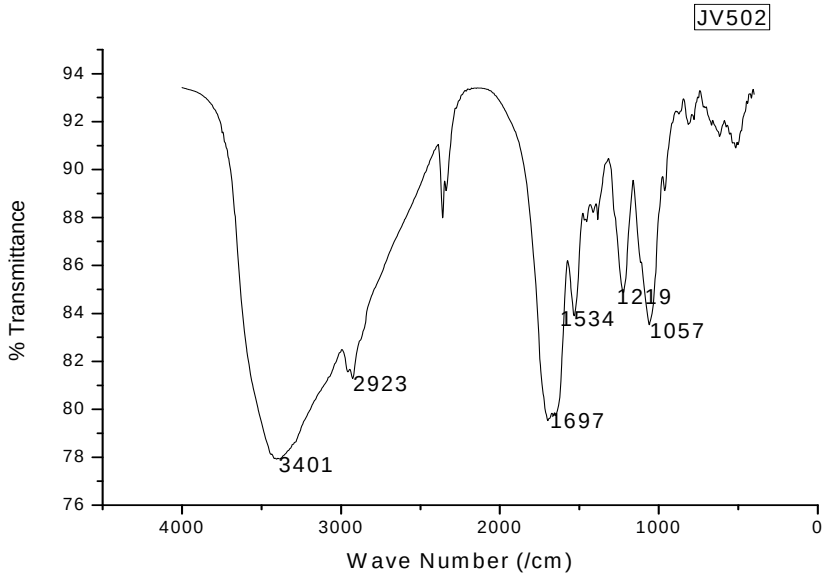


Fig. 27: FTIR result for JV502

Inference

Wavenumber	Bond	Functional group
3401	O–H stretch, H–bonded	Alcohols, phenols
2923	C–H stretch	Alkanes
1697	C=O stretch	Carbonyls (general)
1534	N–O asymmetric stretch	Nitro compounds
1219	C–H wag (–CH_2X)	Alkyl halides
1057	C–N stretch	Aliphatic amines

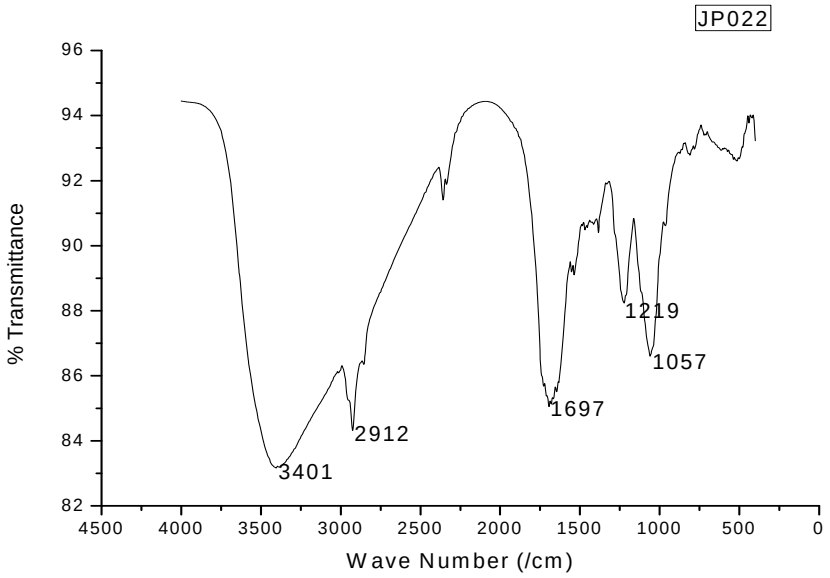


Fig. 28: FTIR result of JP022

Inference

Wavenumber	Bond	Functional group
3401	O–H stretch, H–bonded	Alcohols, phenols
2912	C–H stretch	Alkanes
1697	C=O stretch	Carbonyls (general)
1219	C–O stretch	Alcohols, carboxylic acids, esters, ethers
1057	C–N stretch	Aliphatic amines

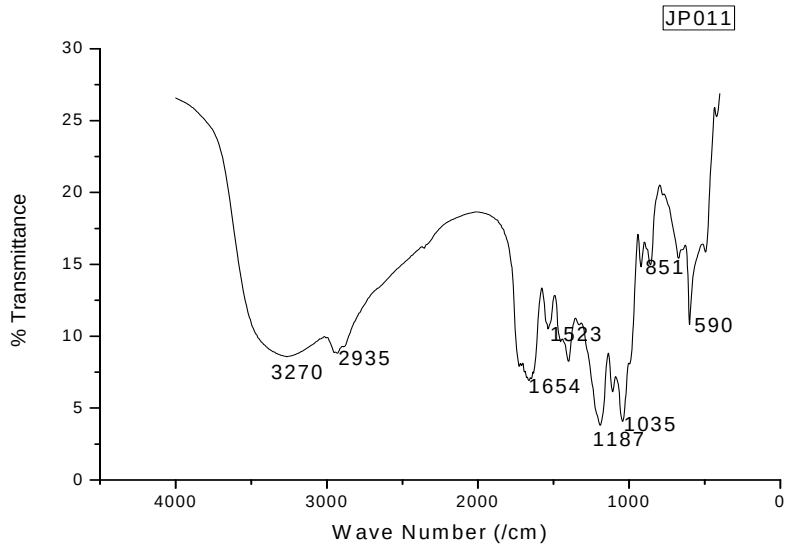


Fig. 29: FTIR result of JP011

Inference

Wavenumber	Bond	Functional group
3270	N–H stretch	Primary, Secondary amines, Amides
2935	C–H stretch	Alkanes
1654	–C=C– stretch	Alkenes
1523	N–O asymmetric stretch	Nitro compounds
1187	C–H wag ($–CH_2X$)	Alkyl halides
1035	C–O stretch	Alcohols, Carboxylic acids, Esters, Ethers
851	C–H "oop"	Aromatics

5.9. Antimicrobial activity

The antimicrobial activities of the biosurfactants produced from 5 strains were seen for 6 pathogenic strains (Fig. 30, Fig. 31, and Fig. 32). These were *Bacillus, Shigella, Streptococcus, Escherichia coli, Proteus and Salmonella.*

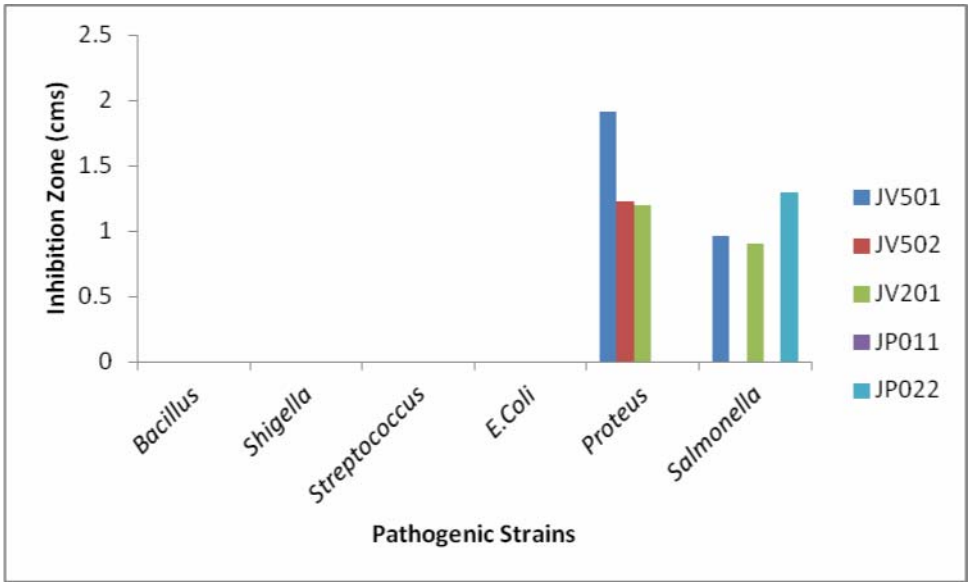


Fig. 30: Antimicrobial activity of crude biosurfactant on the six pathogenic strains

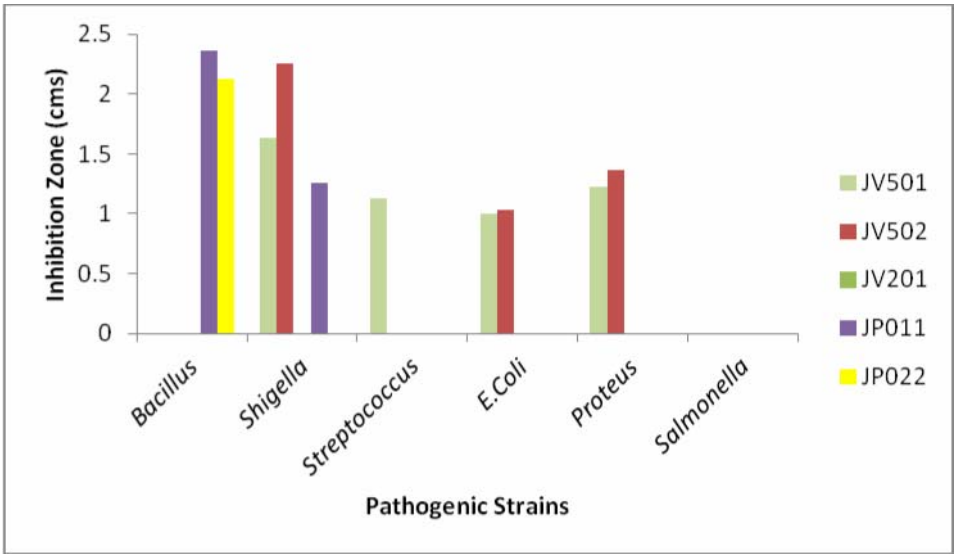
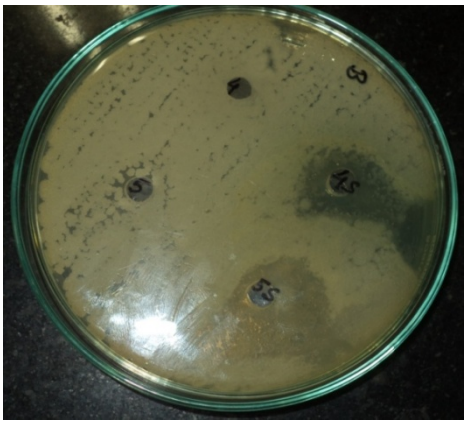
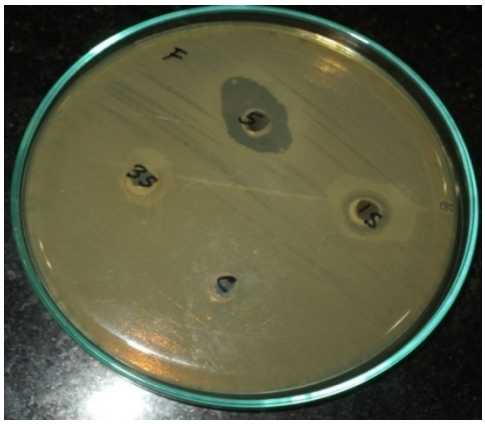


Fig. 31: Antimicrobial activity of Supernatant (biosurfactant) on the six pathogenic strains





A. Positive

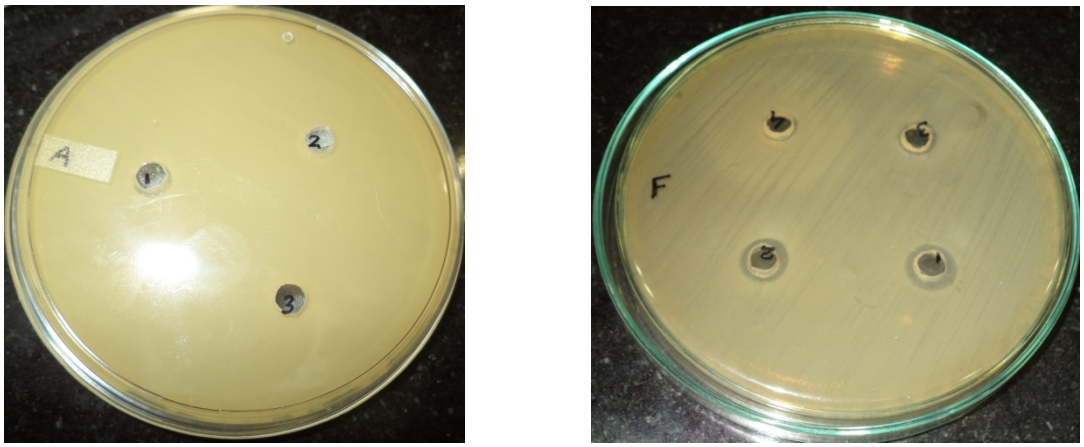


Fig. 32: Plates showing Antimicrobial Activity by the biosurfactant extracted from the strains

5.10. Antioxidant activity test: The biosurfactant extracted were tested for their antioxidant activity showing negative results (Table 11).

Table 11: The O.D. at 517 nm showing negative antioxidant activity

Strain name	Control	O.D. after 30 min	O.D. at 1 h	O.D. after 1.30 h
JV501	1.290	1.434	1.412	1.376
JV502	1.290	1.834	1.757	1.706
JV201	1.290	1.717	1.525	1.480
JP011	1.290	1.760	1.577	1.517
JP022	1.290	1.629	1.457	1.328

5.11. Anti adhesive test

Anti adhesive test against *Bacillus*

Mainly anti adhesive property depends upon the concentration of the biosurfactant and the microorganisms used. Here the crude biosurfactants were extracted from 5 strains. *Bacillus* was taken as pathogenic strain to test the anti-adhesive property of these 5 biosurfactants and PBS (phosphate buffer saline) was used as control that contained no biosurfactant. It was observed that biosurfactant extracted from JV201 showed anti-adhesive value 22.61% (Table 12) for the microorganisms *Bacillus* at minor concentration (1.87mg/ ml) means 22.61% adhesiveness inhibited.

Table 12: Percentage of Anti-adhesiveness against *Bacillus*

Strain name	Biosurfactant conc. (mg/ml)										Control
	50	25	12	7.5	3.75	1.87	0.93	0.46	0.23	0.11	
JV501	15.47	11.90	16.66	9.52	10.71	8.33	10.71	4.76	8.33	14.28	0.084
JV201	-59.52	19.04	21.42	8.33	20.23	22.61	21.42	14.28	10.71	9.52	0.084
JV502	9.52	-7.14	20.23	13.09	17.05	13.09	1.19	9.52	-2.38	-3.57	0.084
JP022	-8.33	4.76	11.9	8.33	14.28	13.09	4.76	-9.52	-17.85	-3.57	0.084
JP011	-126.19	-70.23	-48.80	-25	-22.61	-44.04	-48.80	-29.76	-21.42	4.76	0.084

5.12. Anti adhesive test against *Streptococcus*

Here the microorganism *Streptococcus* that was used for anti-adhesive test. Like above PBS was used as control. Here biosurfactants from JV501.JV201, JP022 were taken to test the anti adhesive property against *Streptococcus.* It was observed that JV201 showed the anti-adhesive value for *Streptococcus* was 23.80% at very low concentration (0.93 mg/ml) and JP011 and JV502 showed anti-adhesive value for *Streptococcus* at 25.89% at 12 mg/ml concentration and 28.94% at 7.5 mg/ml concentration. These were given in the Table 13.

Table 13: Percentage of Anti adhesiveness against *Streptococcus*

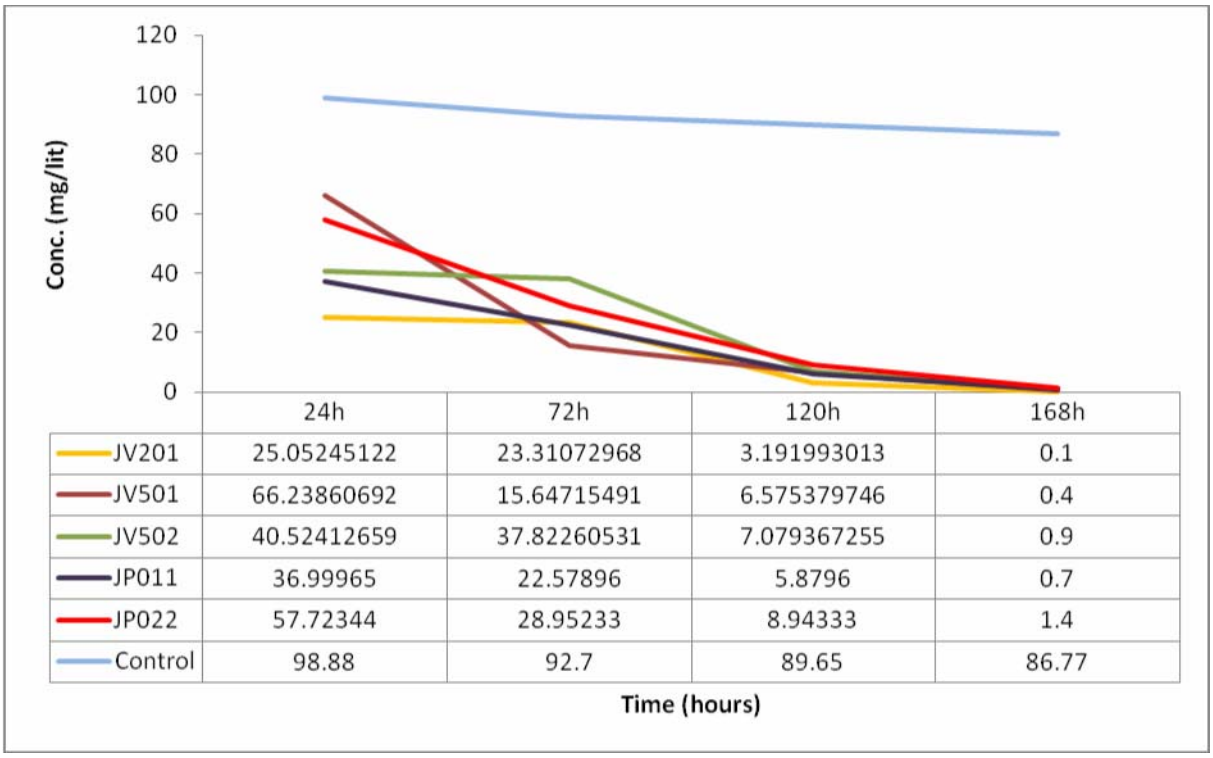
Strain Name	Biosurfactant conc. (mg/ml)										Control
	50	25	12	7.5	3.75	1.87	0.93	0.46	0.23	0.11	
JV501	-16.66	8.33	7.14	3.57	2.38	-8.33	-25	-94.04	-140.4	-129.7	0.084
JV201	-85.71	20.23	21.23	22.61	22.61	22.61	23.8	2.38	-4.76	-40.47	0.084
JP022	-70.23	-26.19	1.19	2.38	2.38	-2.38	3.57	14.28	-13.09	-51.19	0.084
JP011	19.26	8.55	25.89	7.98	6.55	-7.54	-43	-94.8	-2.98	-6.89	0.084
JV502	3.12	6.77	15.28	28.94	18.78	4.89	5.99	3.14	-5.89	-3.29	0.084

5.13. Biodegradation of Polycyclic aromatic Hydrocarbons (PAHS) by Biosurfactant:

5.13.1. Phenanthrene Biodegradation:

Phenanthrene was added to the Bushnell Hass broth in the concentration 100 mg/l for 5 strains- JV201, JV501, JV502, JP011 and JP022. O.D. was taken at 292 nm for 4 days – 1st day, 3rd day, 5th day and 7th day by extracting it with n-hexane. Among these 5

strains, it was observed that JV201 and JP011 very efficiently degraded phenanthrene from initial of 100 mg concentration to 3.19 and 8.94 mg in 120 hours. The other strains also should very good degradation results. (Fig. 33)

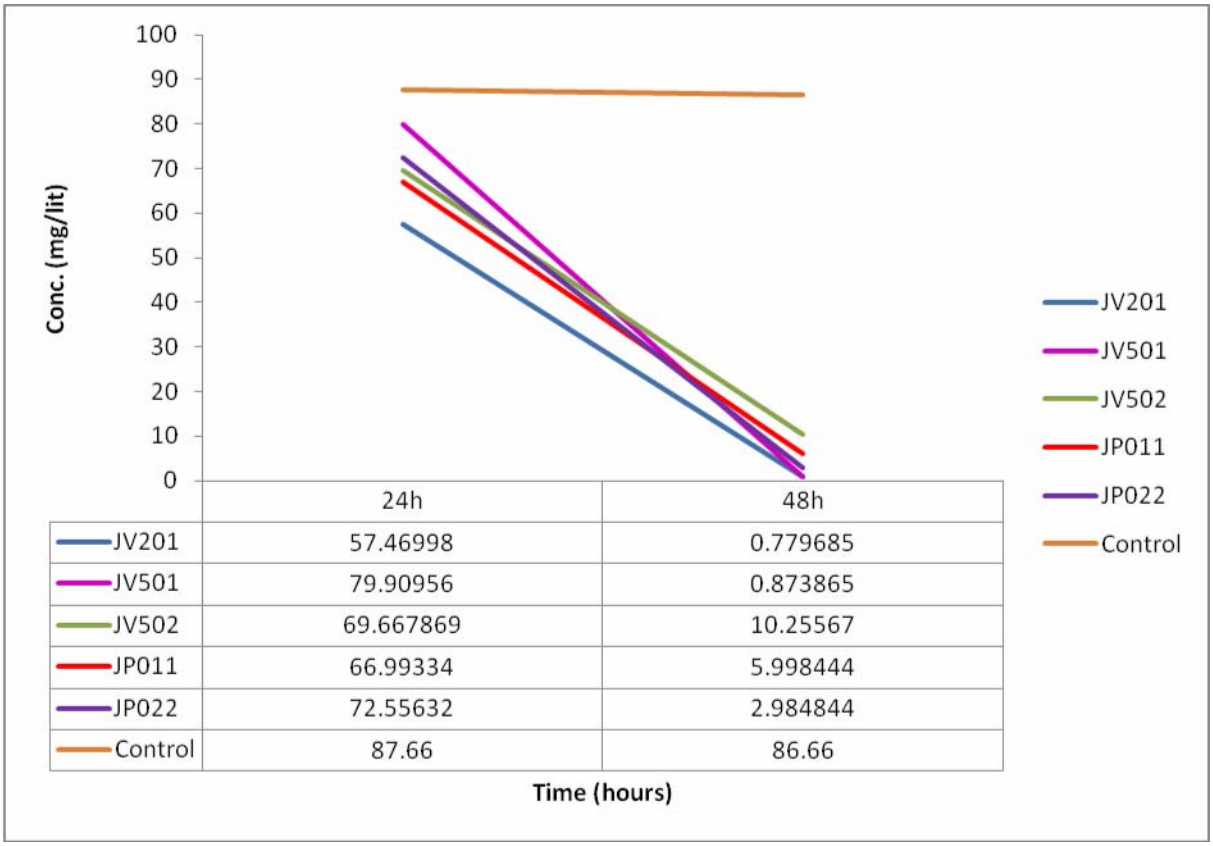


	24h	72h	120h	168h
JV201	25.05245122	23.31072968	3.191993013	0.1
JV501	66.23860692	15.64715491	6.575379746	0.4
JV502	40.52412659	37.82260531	7.079367255	0.9
JP011	36.99965	22.57896	5.8796	0.7
JP022	57.72344	28.95233	8.94333	1.4
Control	98.88	92.7	89.65	86.77

Fig. 33: Isolates showing penanthrene Degradation

5.13.2. Naphthalene Biodegradation

Naphthalene was added to the Bushnell Haas broth in the concentration 100 mg/l for 5 strains- JV201, JV501, JV502, JP011 and JP022. Absorbance were taken at 254 nm for 4 days – 1st day, 3rd day, 5th day and 7th day by extracting it with n-hexane. Among these 5 strains, it was observed that JV201 and JP011 have shown higher efficiency in degradation of naphthalene and strain JV501 showed complete degradation of naphthalene very quickly (Fig. 34).



	24h	48h
JV201	57.46998	0.779685
JV501	79.90956	0.873865
JV502	69.667869	10.25567
JP011	66.99334	5.998444
JP022	72.55632	2.984844
Control	87.66	86.66

Fig. 34: Isolates showing naphthalene degradation

6. Discussion

Fourteen bacterial strains were isolated from Paradeep port, Vishakhapatnam, Rishikulya, Bhitarakanika marine water and streaked in Luria Bertani agar plates for several times and maintained at pure culture. These bacterial strains were screened to check the biosurfactant production.

In oil displacement test, twelve strains were showing the positive results and two strains are showing negative results (JV801 and NP802). In drop collapse method, all strains were showing positive results except JV202 and JV101. In emulsification assay, JP022 showed 41% of emulsification activity and the other strains ranging between 35-40% of emulsification activity except NP202, NP103 and JV101, they were giving negative results. In blood haemolytic test, JP011 showed α haemolysis, JP022- β haemolysis, JV201- β haemolysis, JV501- β haemolysis, ATCC- α haemolysis and others were showing negative results.

From these biosurfactant screening assays, we have seen that five strains named JV501, JV201, JV502, JP011 and JP022 were showing positive results of producing biosurfactant. So these five strains were taken for further study.

The characterizations of these five strains were done in SEM (Scanning Electron Microscopy) and it was observed that all four are rod except JV501 (cocci). From biochemical tests, mannitol motility test, nitrate reduction test and sulphide indole motility tests, it was observed that JV501, JV201, JV502 were showing mannitol fermentation and also motile in nature. Others two were showing negative results in mannitol fermentation and also non-motile. Like this also in nitrate reduction test, JV501 and JV502 showing in reduction of nitrate to nitrite and others were showing negative results. In sulphide production tests all were showing negative results.

The antimicrobial properties of the biosurfactants have been widely reported. However, the biosurfactants with antimicrobial properties reported till date is produced mostly by the micro-organisms of terrestrial origin. However, the number of reports on marine antimicrobial biosurfactant molecules is negligible, so their antimicrobial potentials have not been explored in details. This problem was identified in the present work and the biosurfactants isolated from marine bacteria as well as petrochemical wastes were tested for

antimicrobial action against a battery of pathogenic test organisms. Six pathogenic strains named *Proteus, Bacillus, Shigella, Escherichia coli, Streptococcus, Salmonella* were taken for antimicrobial test. Among these five stains, biosurfactants produced from JV501 showed antimicrobial activity against *Proteus, Salmonella,* JV502 against *Proteus*, JV201 against *Proteus, Salmonella* and JP022 against *Salmonella*. Like this also supernatants of these five strains were taken to test the antimicrobial test. Among these, JP011 showed antimicrobial activity against *Bacillus* and *Shigella*, JP022 against Bacillus, JV501 against *Shigella, Streptococcus* and *Escherichia*, JV502 *Shigella, Escherichia coli*.

Our results illustrated antimicrobial activity and thus can be useful in many domestic and commercial uses. The isolated biosurfactant non-selectively showed activity against both Gram-positive and Gram negative bacterial strains. This is quite contrasting to earlier reports on antimicrobial actions of the biosurfactants where the lipopeptide biosurfactants have been reported to be active mostly against Gram-positive bacteria (Singh and Cameotra, 2004).

The growth pattern of the biosurfactant producing isolates was usually between 16-20 hrs having good biosurfactant production during this time period. The chemical characterization of the produced biosurfactant using FTIR showed that that the peak obtained through this analysis usually corresponds to primary and secondary amines functional groups, also having carboxylic acid stretch, alkane stretch and aromatic groups present. The carbohydrate estimation using Phenol-sulphuric acid test and protein estimation using Bradford assay showed that there was considerable carbohydrate and protein content in the extracted biosurfactant. Different aliphatic and aromatic carbon sources were used as substrate for the growth of biosurfactant producing bacteria to have an optimisation study of which sources are widely utilised (Chukwudi et al., 2012). It has been shown that all five strains could easily utilize both glycerol and sucrose. The growth was also observed in different aromatic compounds like naphthalene, pyrene, phenanthrene, biphenyl, kerosene showing that the bacteria could easily utilize naphthalene and phenanthrene more readily, and then comes biphenyl. The rationale behind biosurfactant production on hydrocarbon utilization should stimulate itself by enhancing the substrate availability. In some literatures it was mentioned that biosurfactant production in presence of hydrocarbons showed better production of biosurfactants (Kumar et al., 2006). Here the result was same; the bacterial strains were showing better production of biosurfactant by utilizing the PAHs as carbon source. Biosurfactants usually lowers the tensioactive force between the two phases. The surface tension of this fraction of the strains JP011, JP022, JV201, JV501, JV502 was found

to be in the range of 40-55 mN/m, the lowest (43.776 mN/m) of strain JP011 indicating its powerful surface tension-reducing property.

The anti adhesive nature of biosurfactant was also tested for the five strains against *Bacillus* and *Streptococcus* of which few strains producing biosurfactant showed good anti adhesiveness. This property can be attributed for cleaning of pathogenic organisms present in medical equipments and efficiently used in medical uses. The antioxidant potential of the biosurfactant was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH method is widely used and the easiest method to determine the antioxidant activity of compounds. But the isolated strains did not show any positive antioxidant results.

Biosurfactant utilised in bioremediation has been harnessed relentlessly for biotechnological purposes. we have isolated and identified five potent isolates having high surface tension reducing property- JV201, JV501, JV502, JP011, and JP022 were identified to be *Ochrobactrum, Streptococcus*, *Pseudomonas* sp., *Pseudomonas aeruginosa*, and *Achromobacter xylosoxidans* having 99.9%, 99.6%, 99%, 99.3%, 98.6% of degradation of phenanthrene (100mg/l) and 99%, 99.1%, 89.75%, 94.01%, 97.02% of degradation of naphthalene (100 mg/l) respectively having good antimicrobial and anti-adhesive properties.

7. CONCLUSION

Now-a-days the production of biosurfactant is increasing due its properties like low toxicity, biodegradability, digestibility and biocompatibility and also due to its vast applications in bioremediation of various toxic substances like PAHs. It is produced on living surfaces mainly microbial cell surfaces or synthesized extracellularly amphiphilic compounds reduces the interfacial tension between the surfaces and interface respectively. When bacteria is present in a stress conditions like hydrophobic environment, they utilize these hydrophobic substances as carbon and energy sources, it produces biosurfactant which helps in conversion of the hydrophobic layer into small micelles which it can easily engulf as a carbon source which is the basic nutritional requirement.

PAHs are released in to the environment by various sources. These can be classified in to natural sources and anthropogenic sources. But anthropogenic sources produce more PAHs than natural sources. The anthropogenic sources are the waste products of various industries (Petroleum, Diesel), domestic sewages, oil spill in marine environment, smoking, by burning of coal, diesel, and petrol (fuel for energy) .The hydrocarbons contaminate the subsoil and groundwater. It enters in to the food chain and disturbs it. Some of the light poly aromatic hydrocarbons bind to the dust particles in atmosphere and persist for long time. It enters in to the human body through inhalation, food, skin and cause mutagenic and carcinogenic effects. As these are highly toxic, if even low amount of them present in the soil, causes serious problems.

Eco-friendly technologies must be used to clean the environment such as degradation by microorganisms. Bioremediation has been accepted as an important method for the treatment of oil pollution by biosurfactant produced by bacterial colonies. Under certain conditions, living microorganisms primarily bacteria can metabolize various classes of hydrocarbons compound. Since hydrocarbons contain high organic matter, it can be assimilated by the bacteria as a carbon source. There are many techniques used to clean up the organic contaminants. Some non-biological methods such as excavation and disposal of contaminated soil to landfill sites are used. Biological methods are the processes that use plants (phytoremedation) or microorganisms (bioremediation) to remove these pollutants from soil. Therefore, utilising biobased techniques like production of biosurfactant in large quantities through bioreactors can be efficiently commercialized in industries and can be applied in highly polluted sites for complete biodegradation of the toxic polycyclic aromatic hydrocarbons.

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9. APPENDIX

The media used and their compositions are given below

A) Media

i) Luria Bertani Media

Components	Quantity (g/l)
Tryptone	2.00
NaCl	1.00
Yeast Extract	0.5%
pH (at 25 ˚C)	7.0

ii) Bushnell Haas Broth (BHB)

Components	Quantity (g/l)
Magnesium sulphate	0.20
Calcium chloride	0.02
Monopotassium phosphate	1.00
Dipotassium phosphate	1.00
Ammonium nitrate	1.00
Ferric chloride	0.05
pH (at 25 ˚ C)	7.0 ±0.2

iii) Muller Hinton Broth (MHB)

Components	Quantity(g/l)
Beef infusion solids	4.0
Starch	1.5

Casein hydrolysate	17.5
pH (at 37 ˚C)	7.4± 0.2

B) Strains

Bacterial isolates were stained by using Gram's staining methods

Ingredients	Uses
Crystal violet	Primary staining Agent
Safranin	Secondary Staining Agent
Lugol's Iodine	Mordant
Acetone	Decolourising Agent

C) **Polycyclic aromatic Hydrocarbons**

Used polycyclic aromatic hydrocarbons are: - Phenanthrene, Naphthalene, Pyrene, Biphenyl.

These are taken in the concentration of 100mg/l